BIOCHEMICAL AND PATHOPHYSIOLOGICAL STUDIES OF FISH PARASITES *ADENOSCOLEX OREINI* AND *POMPHORHYNCHUS KASHMIRENSIS*

THESIS

SUBMITTED FOR THE DEGREE OF

Doctor of Philosophy

IN

ZOOLOGY

By

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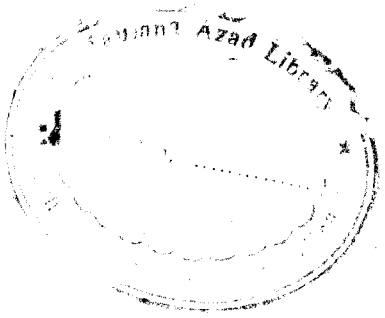
SECTION OF PARASITOLOGY
DEPARTMENT OF ZOOLOGY
ALIGARH MUSLIM UNIVERSITY
ALIGARH - 202002 (INDIA)

March, 2005



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INDIA

Sections :
1. AGRICULTURAL NEMATOLOGY
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3. FISHERY SCIENCE & AQUACULTURE
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CERTIFICATE

This is to certify that the thesis entitled "**Biochemical and Pathophysiological Studies of fish parasites *Adenoscolex oreini* and *Pomphorhynchus kashmirensis***" submitted by **Mrs. Yasreel Mustafa** embodies original work, done by the candidate herself. The entire work was carried out under my supervision and that I allow her to submit the same in the fulfillment of the requirements for the degree of **Doctor of Philosophy** in Zoology of this University.

Dr. Malik Irshadullah
(Supervisor)

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ACKNOWLEDGEMENTS

I wish to express my deepest appreciations and sincere gratitude to my supervisor Dr. Malik Irshadullah for his guidance, encouragement, inspiration, unstinted support and aesthetic suggestions throughout the course of present investigation.

I am deeply indebted and thankful to Prof. M. Hayat, Chairman, Department of Zoology, A.M.U., Aligarh for providing the necessary laboratory facilities.

It gives me immense satisfaction and pleasure to extend my humble gratefulness to Prof. A. H. Siddique for his encouragement and valuable suggestions. The help rendered by Prof. H. U. Farooqi is sincerely acknowledged. I am also thankful to Dr. Mukhtar. A. Khan for his support and encouragement.

My deep regards and honour are due to all the staff members of NIAE and Fisheries Department, J&K, for providing me necessary laboratory facilities during my collection in the state. I am also thankful to fishermen of valley at various sites who provided an aid in collection of fish.

My warmest thanks to Dr. Andrabi and Dr. Mustafa of University of Kashmir, for providing me help during scanning electron microscopy. I am also thankful to Mr. Fazal-ur Rehman Khan, Department of Agriculture, A. M. U. for helping me in microphotography of slides. I extend thank to Mr. Ishtiyaq Ahmad Shah for his valuable help in sample handling. The help rendered by Mrs. Kavita Singh in electrophoresis is gratefully acknowledged.

I express my special thanks to my colleagues, Muheet Alam and Shweta, Shoeb and Sana whose help and cooperation endeavour the success of present work. I am also thankful to Mr. Shakir Ali and Mr. Ramesh Chandra for there support and to Mr. Mujtaba for his help in photography. My thanks

are also extended to Dr. S. M. Ali Badruddin, Mr. Rais A. Khan and Mr. Shakeel for preparing graphs, plates and typing to make the thesis presentable.

It is an unvarnished truth that the treatise would have never materialized without the help of my father who not only encouraged me but also provided assistance in every way during the course of work and my mother who appreciated my instinct. I wish to express my heart-felt gratitude to my grand parents, parents and in-laws whose constant encouragement, help, good wishes and blessings have always been the source of inspiration throughout my work. My thanks are also extended to my local guardians who provided me encouragement and moral support. I am also highly thankful to Dr. Asif, Mr. Ubaid, Miss Lubna, Miss Naheen, Miss Tousia and other family members for there encouragements and support.

Words fail to express my sincere regards and gratefulness to my husband Dr. Arif Iqbal Vakil, whose self-denying love, encouragement and support made it possible for me to accomplish this work.

Yasreel

YASREEL MUSTAFA

INTRODUCTION AND STATEMENT OF PROBLEM

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India is basically an agrarian country where almost 70% of the human population depends on Agriculture for livelihood **(Delphine and Thatheyus, 2003)**. Agriculture sector provides 26% of Gross Domestic Production (GDP), 64% employment and accounts for 18% of India's exports **(Anon, 1995)**. Since independence, there has been a four-fold increase in food grain production. The milk and egg production increased by 2.5 and 3 times during the last two decades. Thus, India has moved from chronic food deficit to a surplus and is now becoming a major producer and consumer of a wide range of agricultural, fish and other products.

Over 90% of world's living biomass is present in the aquatic water bodies, which cover about 71% of the earth's surface. The marine sources provide about 20% animal proteins for human consumption. India has a vast potential of fishing resources comprising 7,517 km of coastline, 29,000 km of rivers, 1.7 million hectares of reservoirs, 0.902 million hectares of brackish water areas and 0.753 million hectares of tanks and ponds **(Raman and Balaguru, 1990)**. These authors further reported that the catch of inland and marine fishes increased to about 5 million tones. In India, the inland fisheries are very rich and contribute about 30% of the total fish production. The fresh-water fisheries comprise the great river systems, lakes, irrigation canals, tanks, ponds and reservoirs. The cold-water fisheries include the hill streams, lakes

and man-made reservoirs. The severe cold-water bodies are present in Jammu and Kashmir, Uttar Pradesh, Sikkim, Bihar, Kerala, Arunachal Pradesh, etc. These cold-water bodies are fed by streams, rain water and melting snow. The fish present in these water bodies have low temperature tolerance (0-4°C), which include *Salmo* spp, *Schizothorax* spp, *Labeo* spp, *Noemacheilus* spp, *Tor* spp, *Glyptothorax* spp, etc. In India the fisheries potential remains largely under-exploited as compared to that of food crops. The implementation of small scale occupational majors in these sectors will sort out many problems of the country. It will feed the future generations without degrading our resources and will make us independent and self sufficient, leading to enhance the economy of the country. The fisheries sector plays an important role in Indian economy by contributing to national income, exports, food, nutritional security and employment **(Inception Report, 2001)**. In Asia, about one billion people rely on fish as their primary source of protein. At present it has been estimated that fisheries provide employment to about six million people's world wide **(FAO, 2000)**. The fisheries sector continues to be a thrust area of India's development programs due to its vital contributions.

Presently the reliance on fish as a primary source of food has rapidly increased resulting in the development of better strains of fishes. The fish is an excellent source of animal protein which has relatively high digestibility. The fish proteins have many essential amino acids in desirable amounts and hence account for high biological value. Besides this, carbohydrates, vitamins (A, D,

and E), iron, calcium and other minerals are also present in fish flesh. Although India has enormous fisheries resources but the productivity is very low as compared to other countries of the world, which may be due to many factors. Parasitic infection is one of the most important factors which causes considerable damage to the fish and sometimes mortalities may also occur leading to great economic losses.

Fish by virtue of their geographical history, ecological situation and physiological specialties harbour many parasitic infections. Generally, fish act as the definitive hosts of many parasites as well as act as intermediate host and harbour the larval stages. The disease dynamics of fish in captivity differ from those inhabiting natural environments. A parasitic disease is the result of the imbalance between the resistance capacity of the host and pathogenic force of many aggressive factors (Fig.1). In India, **Pal (1982)** has identified various environmental factors, which are responsible for parasitic diseases among fishes. Negative variations of environmental parameters are sometimes sufficient to weaken the host and help the development of parasite. Modifications of those factors, which are responsible for various parasitic diseases is difficult in open areas and in some cases often impractical. Thus, the only means of intervention in many cases is through knowledge of the pathogen. The parasitic fauna of the fishes includes protozoans, monogeneans, digeneans, cestodes, nematodes, acanthocephalans and few molluscs besides viruses and bacteria. The parasitic diseases provide a considerable threat to the

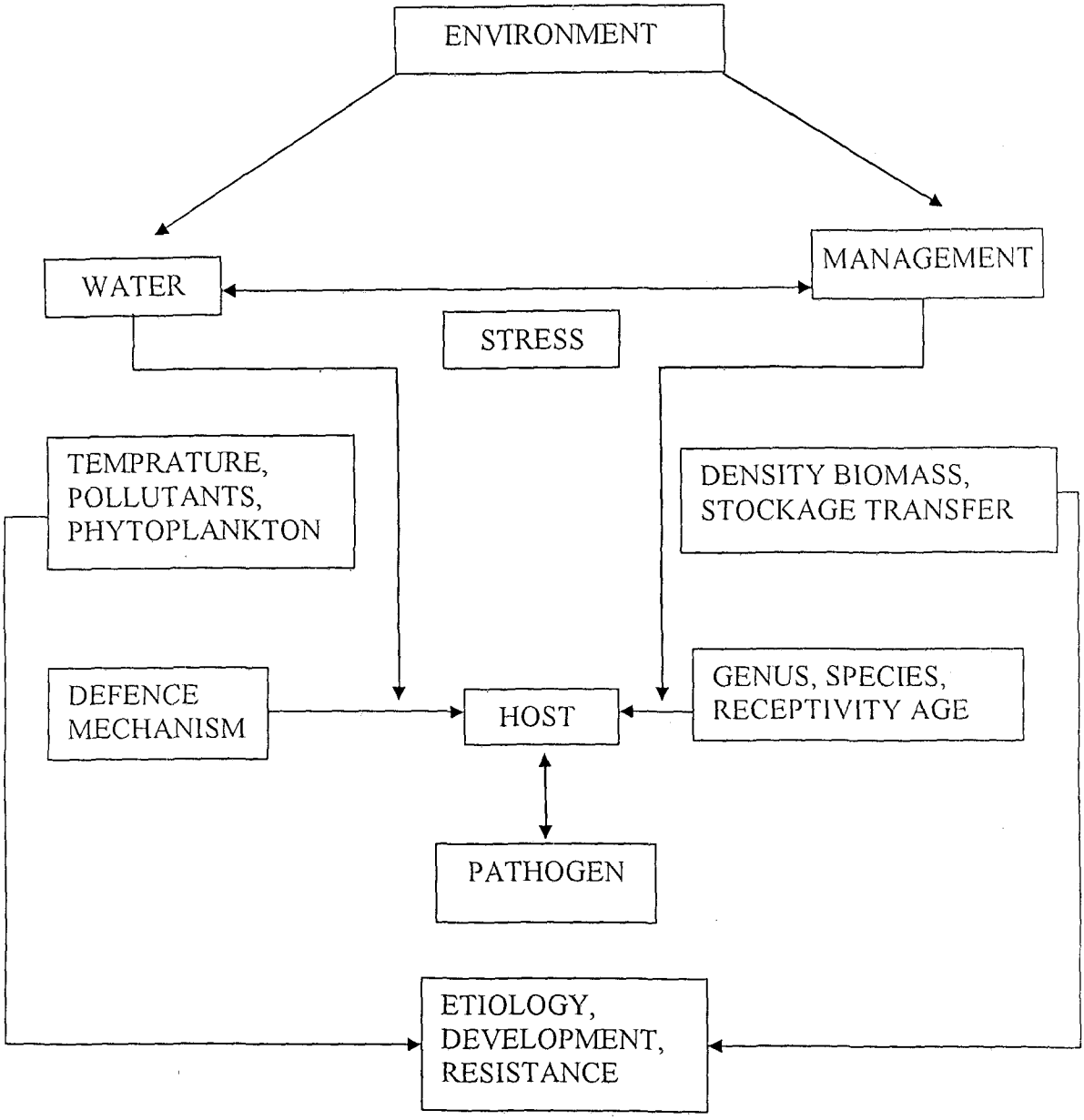


Fig. 1. Interaction between host and pathogenic factors.

fish industry and act as one of the high risk factors for the survival and health of the fishes **(Sindermann, 1986)**. Therefore, in order to increase the productivity of the fishes, the parasitic infections must be controlled. The basic aim of any study related to parasitological problems is to contribute directly or indirectly towards the control of infection. An efficient control of the disease depends on the correct and integrated application of several control measures as shown in Fig. 2.

Among various helminth infections, *Adenoscolex oreini* and *Pomphorhynchus kashmirensis* are the most common parasites found among the fishes of Kashmir valley. These parasites infect *Schizothorax* as well as other cold-water fishes, which also harbour many trematodes and nematodes as well. The incidence of these parasites vary with the seasons of the year **(Dhar and Peerzada, 1989; Chishti and Peerzada, 1998)**. Due to heavy worm burden, the intestine gets almost blocked and thereby belly becomes protruded.

A. oreini is a caryophyllidean cestode found in the intestine of fresh water fishes and uses many annelids (*Tubifex* spp.) as its intermediate hosts. The complete life cycle of this parasite is shown in Fig. 3. This parasite is elongated, dorsoventrally flattened with crenated margin posteriorly (Plate 1).The pathology caused by the tapeworms in the gut is tissue alteration, destruction, mechanical blockage and malabsorption. Large numbers of parasites reduce the lumen diameter by more than 50% and thus affect the movement of food through the intestine **(Shostak and Dick, 1986)**. The

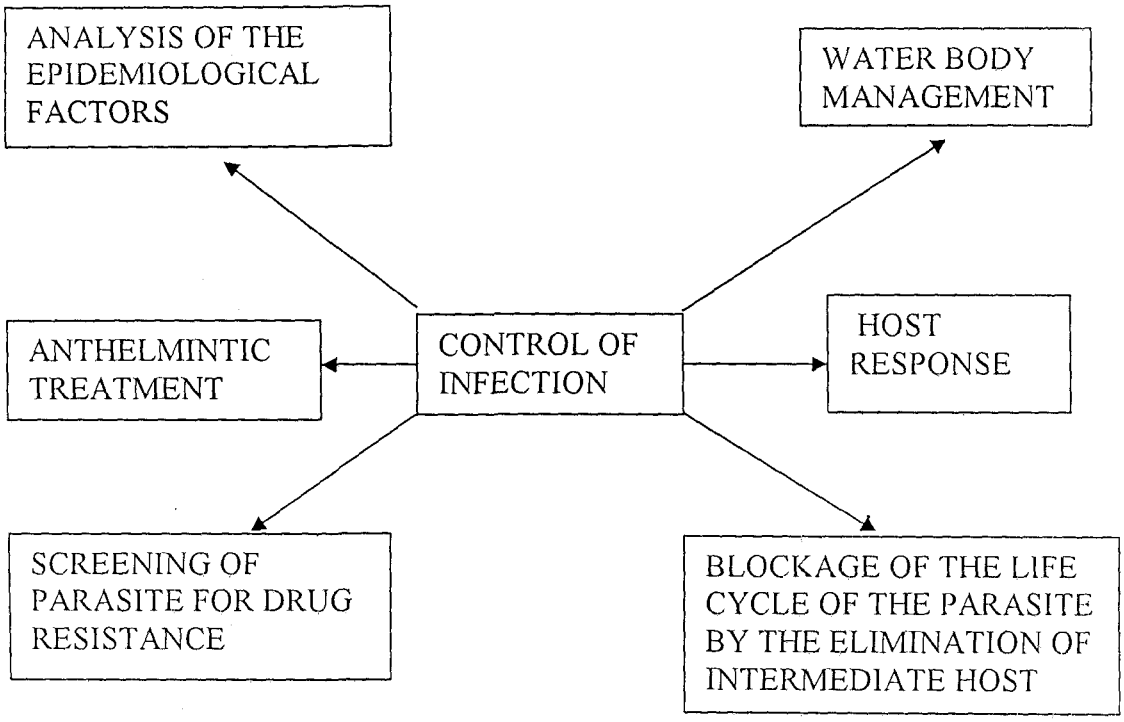


Fig. 2. Strategies of parasite control.

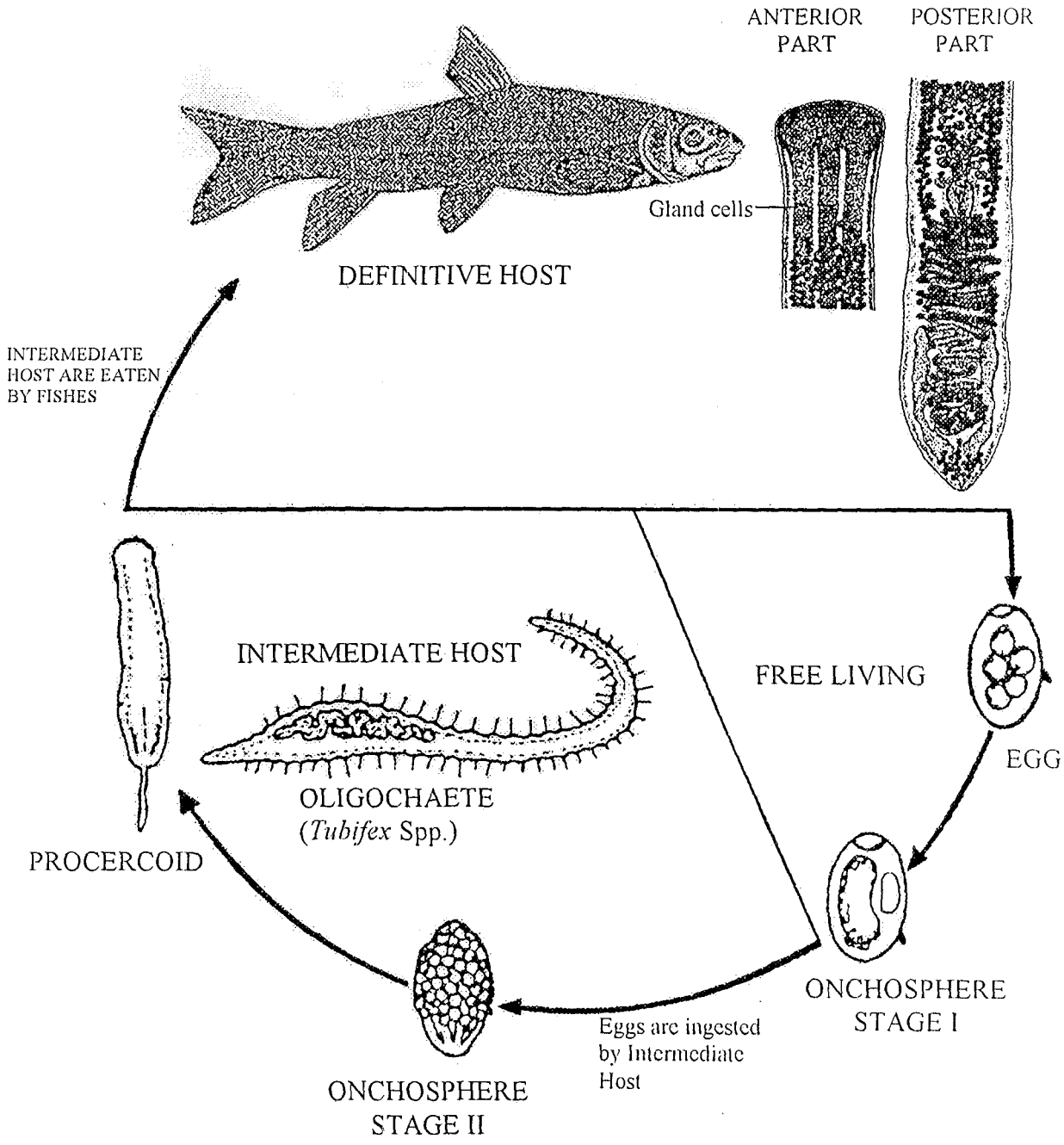


Fig. 3. Life cycle of *Adenoscolex oreini.*

Plate 1. Adult *A. oreini* collected from naturally infected *S. esocinus* of the river Jehlum.

Figs.

A. Adult *S. esocinus.*

B & C. Freshly isolated worms from *S. esocinus.* The coin gives an idea of magnification.

D. Scolex region showing gland cells (arrows), stained with Borax carmine.

E. Middle part of the body showing extension of gland cells (arrow).

F. Posterior region of the body showing crenated margins (arrow).

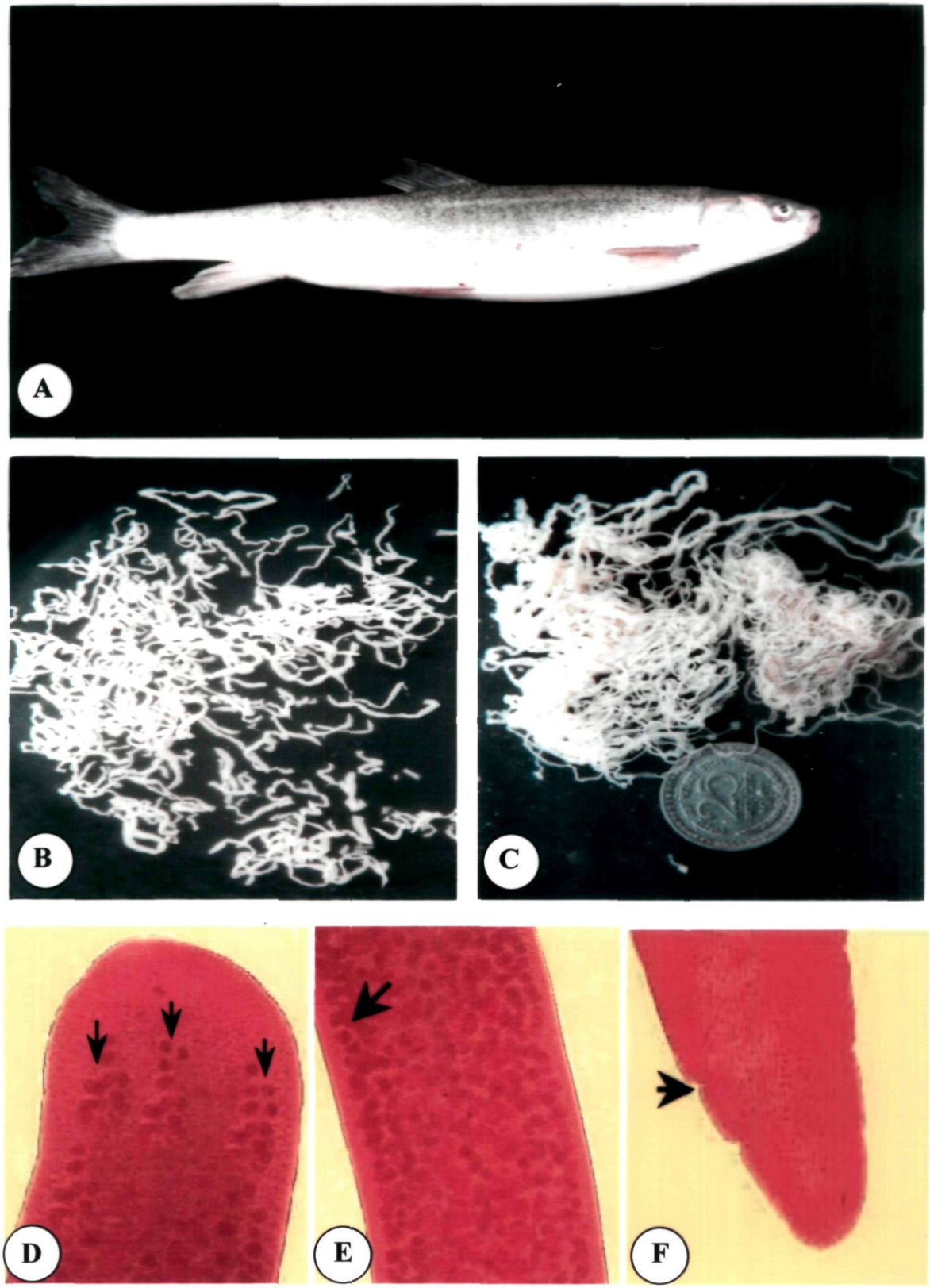


Plate 1

clinical signs that appear due to this parasite are emaciation and anaemia (Scott and Grizzle, 1979). The nutritional demands made by gut tapeworms may adversely affects growth and fitness of the host.

In comparison to cestodes, the acanthocephalans cause much more damage to the fishes. Among acanthocephalans, ***P. kashmirensis*** is one of the most important parasites from economic point of view. This parasite is small having characteristic bulbous neck, long proboscis armed with hooks and shows sexual dimorphism (Plate 2). The females are robust and more in number than males. The life cycle of this parasite is complex involving both vertebrate and invertebrate hosts and is shown in Fig. 4. The adult worms are found in the ileum of larger fishes, which act as definitive host. The amphipod *Gammarus pulex* acts as intermediate host. The eggs containing mature acanthor pass out with the faeces of definitive host and are immediately infective to the intermediate host. On ingestion by the amphipod host the first larval stage acanthor is liberated by the splitting of shell and membranes which then perforates the gut wall by means of its rostellum and reaches into the haemocoel, where it develops into second larval stage acanthella by elongation and differentiation of acanthor. The proboscis and other organs develop which resemble the adult but it is not infective until it rounds up and becomes surrounded by a membranous envelope. At this stage it is known as cystacanth. The cystacanth remains dormant in the tissue of amphipod until it is ingested by the definitive host where it excysts, everts the proboscis and attaches to

Plate 2. Adult *P. kashmirensis* collected from naturally infected *S. esocinus* of the river Jehlum.

Figs.

A, B & C. Intestines of infected *S. esocinus* showing attached *P. kashmirensis* (arrows), note the host's reactions at the sites of attachment (arrow heads). The coin gives an idea of magnification.

D. Male and female *P. kashmirensis*, note the size of the worms. Inset: proboscis at higher magnification showing hooks (arrows).

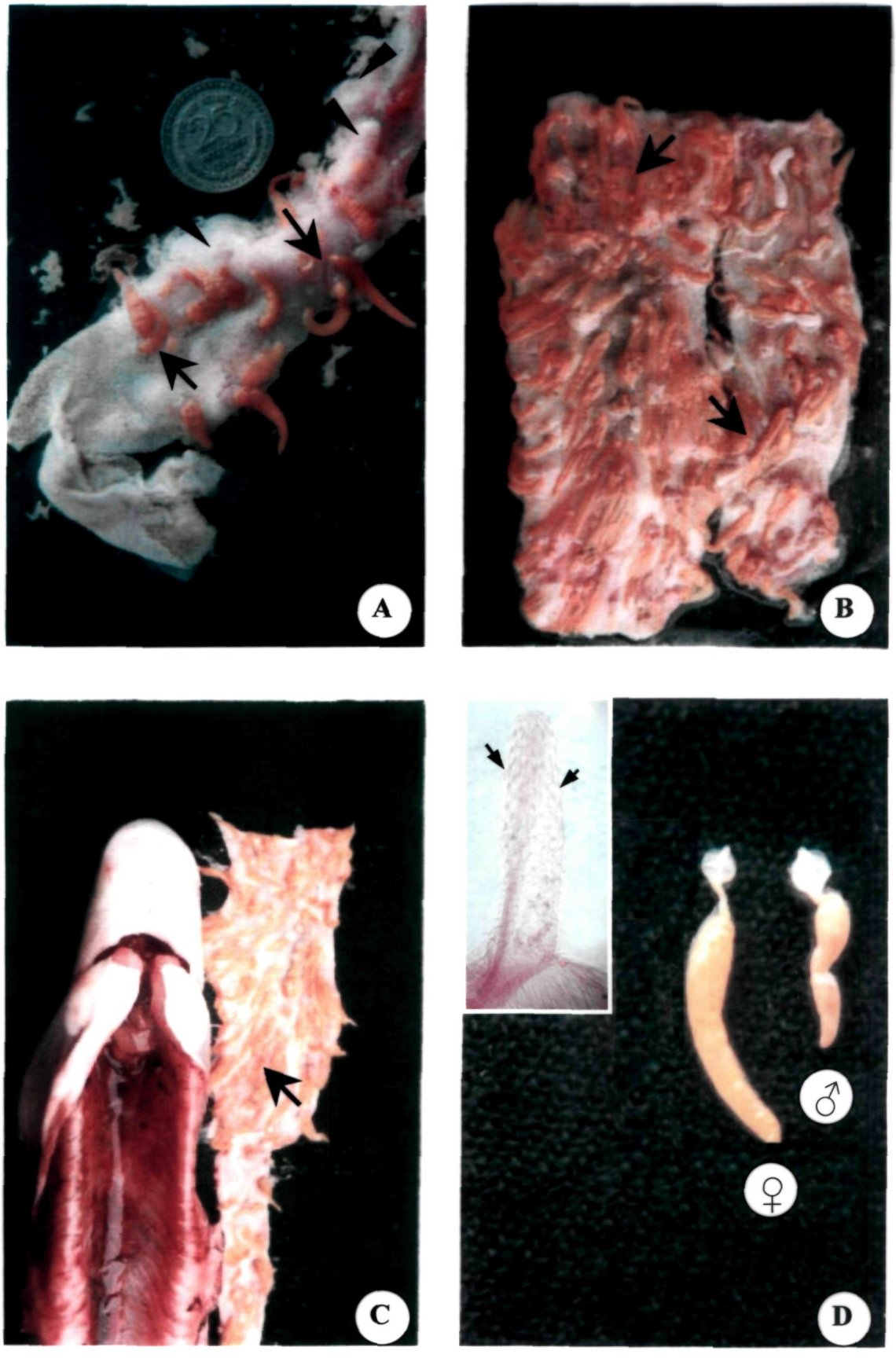


Plate 2

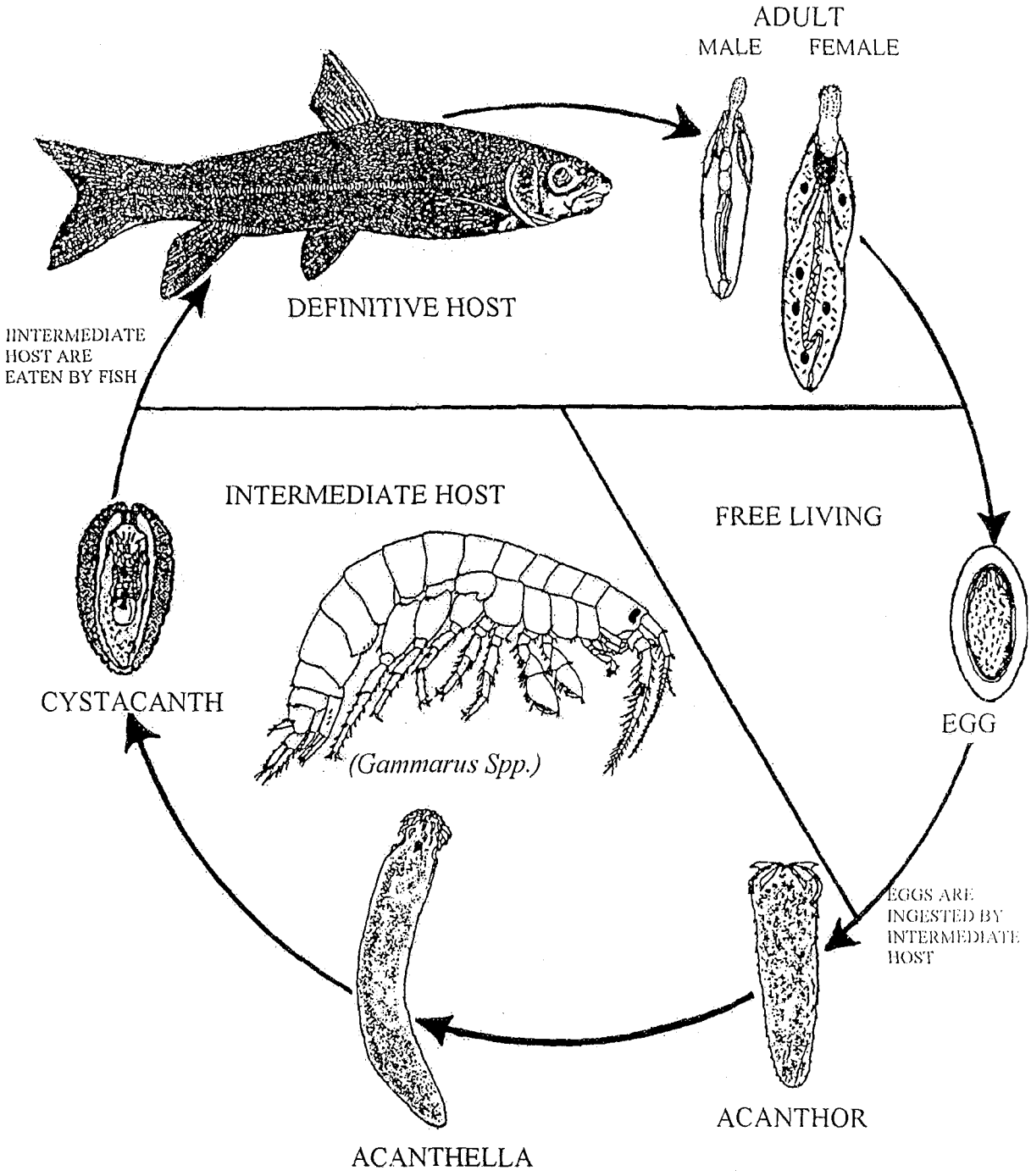


Fig. 4. Life cycle of *P. kashmirensis*.

intestinal wall and hence become adult. In some species of *Pomphorhynchus*, smaller fishes act as paratenic host, where cystacanth emerges and re-encysts in the tissue. The infected fishes with heavy worm burden have swollen abdomen. The parasite may attack the internal organs by boring the intestinal wall and therefore causes more damage to the host. In heavy infection, sub serosal nodules develop in the gut at the point of attachment of the proboscis due to granulomatous reaction. The penetration of proboscis in the serosa causes peritonitis. **Dezfuli (1991)** reported the formation of capsule around the neck and proboscis of ***P. laevis*** due to cellular infiltration in the fibrotic tunnel, which finally protrude into the coelomic cavity.

Recognizing the importance of fish industry in India and economic losses caused by the parasites an immediate attention is needed to control the parasitic infections among fishes. Most of the studies carried out on fish parasites are confined to the taxonomy and biology of the parasite but the epidemiology, biochemistry, pathophysiology and immunology have received little attention. Therefore, emphasis must be given on these lines to prepare a data base information bank of the host-parasite system so that the economic losses caused by the parasites can be minimized. For the eradication and effective control measures, it is necessary to investigate the various epidemiological factors, which determine the frequency and distribution of the disease. In India, the actual epidemiological factors for the prevalence of *Adenoscolex* and *Pomphorhynchus* have not been examined in detail.

Therefore, it is essential to identify various epidemiological factors, which are responsible for the prevalence and transmission of these parasites. This basic data will help in designing an effective epidemiological control measure by interrupting the mode of transmission. Thus, the main objectives of the epidemiology are similar to those of ancient Greek medicine, described by Hippocrates in his book "Epidemics" as "declare the past, diagnose the present and foretell the future **(Jones, 1923)**.

Studies on the protein content and protein profile of *A. oreini* and *P. kashmirensis* highlight the existence of antigenic variabilities and similarities that could be exploited for immunodiagnostic and immunoprophylactic measures by using various immunological tools. Immunological studies to elucidate the relationship between different parasite species, provide meaningful results due to the presence of specific antigenic determinants **(Sinclair, 1970; Clegg and Smyth, 1978; Mitchell and Anders, 1981)**.

It is an established fact that for designing an effective chemotherapeutic or immunological control measure a clear understanding of host-parasite relationship is needed. In this context **Read (1972)** has pointed out that in order to understand such relationship, the two organisms should not be considered as separate entities but as single biological unit. During the successful establishment of the parasite in their habitat, a chain of dynamic interactions particularly pathophysiological and immunological events occur at the host-

parasite interface. Comparatively very little work has been carried out on the immunological response of fish to the parasite. In this connection **Thomas and Woo (1995)** have stated that this may partly be because parasitologists with more classical training do not feel comfortable about using immunological approaches and techniques. However, these techniques can help us better understand the host-parasite relationships, ability of the host to resist infection and to resolve the phylogenetic relationships between species or groups of parasites. The parasite induced pathological and immunological events at the interface would provide an insight in the disease manifestation. Therefore, investigation of some diagnostic pathological markers and antigenic variations are needed which will be certainly helpful for diagnosis of the infection.

The identification, isolation, purification and characterization of parasite antigens are required for vaccination, immunodiagnosis, analysis of immunopathology and for the quantitation of various immune responses in the infected, vaccinated or naturally resistant hosts. Antibodies to defined antigens are required as parasite typing reagents, and also for the analysis of antigenic variability in parasite populations. It has been pointed out that without defined antigens or antibodies, little progress can be made in the dissection of the immunological aspects of the host-parasite relationship.

Antigens have been broadly classified as 'natural antigens' and 'novel antigens' (**Anders *et al.*, 1982**). Natural antigens are those which induce readily detected immune responses under circumstances of natural infection or

vaccination of natural hosts, whereas novel antigens are those which induce immune response in unnatural or non-permissive host. Analysis of novel antigens provides information regarding the gross picture of antigen-antibody reaction. Though the use of total tissue extracts for antigen analysis is of limited value for the studies related to vaccine development but it will provide an overall view of the antigenic heterogeneity or antigenic polymorphism which may help to identify the various parasite species and also to establish the intricate host-parasite relationship. Once the species specific antigens are characterized, they could be used for further studies on immunodiagnosis.

In order to eliminate any parasitic infection, the last programme is chemotherapy. Therefore, there is an urgent need to find out an effective chemotherapeutic agent against these fish parasites. In the present study, the efficacy of various anthelmintics belonging to different groups has also been studied.

Thus, in the light of the above context, the present work has been carried out which may provide a key to the integrated control programme and thereby helping the commercial viability of the fisheries in our country. Knowledge of the parasite biochemistry may help to investigate the target for the drug action and therefore for designing an effective control programme.

HISTORICAL REVIEW

HISTORICAL REVIEW

It is evident from the available literature that the study of helminth parasites of freshwater fishes is an attractive field for parasitologists. Earlier the taxonomy of the parasites attracted the attention of workers until **Dogiel** (1961; 1964) put forward ecological concept of parasitism. He discussed in detail the influence of physical factors, such as water quality, temperature, habitat and biological factors like age, size, sex and migration of the fish host on the parasitic fauna. **Dogiel's** reviews laid the foundation for the study of the ecology of fish parasites. After that many investigators like **Bauer and Stolyarov (1961) and Pugachev (1983)** continued to concentrate on parasitocoenosis whereas, some workers have shifted their work on population dynamics **(Chubb, 1964a; b; Anderson, 1974; 1978; Kennedy, 1977; 1985)** and biology of fish parasites **(Mackiewicz, 1981; Belghyti *et al.*, 1994)**, but the physiology, biochemistry, pathology and immunology were not investigated properly and only scanty information are available.

[I] Prevalence of *Adenoscolex* and *Pomphorhynchus* in the fishes of Kashmir:

The parasitic fauna among fishes of Kashmir remained little known until early forties when **Kaw (1941)** reported *P. kashmirensis* and other species of *Pomphorhynchus* from freshwater fish species *Noemacheilus kashmirensis*. *A. oreini* was first reported by **Fotedar (1958)** from the gut of

Oreinus sinuatus. **Gupta (1967)** published a classified list of helminthic fauna from the fishes of Kashmir including *A. oreini* and *P. kashmirensis*.

A. oreini is a small monozoic caryophyllidean cestode with poorly differentiated scolex and having only one set of reproductive organs. Caryophyllids generally parasitize the freshwater fishes mainly of the families Cyprinidae, Siluridae and Catastomidae **(Yamaguti, 1934)** and uses an oligochaetes (*Tubifex*) as intermediate host **(Szidat, 1938)**. **Mackiewicz (1981)** reviewed the life cycle, zoogeography, evolution, classification and host-parasite relationship of caryophyllidean cestodes in detail. The caryophyllidean tapeworms have wider host spectrum because of feeding habits and diet of the hosts **(Mackiewicz, 1972; Tierney, 1994)**. **Berger and Esch (2002)** reported that the number of host species infected by the parasite was positively related to distribution and abundance of the host. Further, it was shown that the prevalence of helminth parasites including a number of cestode species of the family Caryophyllaeidae in the freshwater fishes varies with the season (reviewed by **Chubb, 1982**). **Lawrence (1970)** studied the effects of seasons, age and sex of the host on the prevalence of endohelminths in *Catostomus commersoni* and reported that there was marked seasonal variation in their incidence. The caryophyllidean cestodes *Glaridacris laruei, G. catostomi* and *Isoglaridacris bulbocirrus* were most abundant during January to April and the intensity of infection increased with the age of the host **(Chubb, 1964a; Lawrence, 1970)**. Many workers reported heavy infection of *Caryophyllaeus*

laticeps during the spring and early winter and suggested that this was due to comparatively high water temperature in the lake during winter (See references in **Kochva, 1967**). During winter the water temperature of the lake increases due to presence of hot springs and therefore the infection of *C. laticeps* increases. **Aydogdu *et al.*, (2003)** studied the seasonal variations and effects of size and age of the host on the prevalence and abundance of parasitic fauna in the wild population of ***Cyprinus carpio*** from Iznik Lake in Turkey. They reported significant differences in the abundance of *C. laticeps* during different seasons indicating a temperature dependant response whereas, age and size of the host did not influence the parasite population. **Anderson (1976)** has shown that the seasonality of the parasite population size was due to the combined effect of temperature dependant mortality rates and fluctuations in the host feeding activity which control the immigration rate of the larval parasites. **Kennedy (1968; 1969a)** and **Anderson (1974)** recorded an increase in population size of *C. laticeps* in the *Leuciscus leuciscus* and *Abramis brama* during the spring season. Recently, **Khan *et al.*, (2004)** reported seasonal changes in the prevalence of *A. oreini* from Dal Lake of Kashmir.

Pomphorhynchus is a common acanthocephalan parasite inhabiting the alimentary canal of fresh and marine water fishes. A number of species of this parasite have been reported from different parts of the world. Various aspects of this parasite have been reviewed by many workers (**Amin 1985; 1987a; Chubb 1982**). Among various species, ***P. kashmirensis*** has been reported from

freshwater fishes of Kashmir **(Kaw, 1941; Fotedar and Dhar, 1977)**. Literature survey clearly reveals that only few workers have made an attempt to find out the incidence and seasonal occurrence of *P. kashm*irensis from lentic (standing) water bodies **(Dhar and Majdah, 1987; Chishti and Peerzada, 1998; Khan *et al.*, 2004)** but no information is available on lotic (running) water bodies. A total of 7.2% fishes have been reported to harbour *P. kashmirensis* and *Neoechinorhynchus manasbalensis* from the Wular Lake of Kashmir, in which *P. kashm*irensis was found predominant **(Chishti and Peerzada, 1998)**. Further, they reported that the incidence varies with the season and species of the fishes. Very high (maximum percent) infection was reported during spring season and *Orienus plagitomus* was found to harbour maximum infection.

The other most studied acanthocephalan species is *P. laevis*, which is found in freshwater (cold and warm) and marine fishes. Three biologically distinct strains of *P. laevis* have been reported from the British Isles which differ in the distribution and host specificity **(Kennedy *et al.*, 1978; Kennedy 1984; Brown 1987)**. It has been reported that the parasitic fauna of freshwater fishes are influenced by water temperature however, the parasites of marine fishes do not fluctuate with the seasons (reviewed by **Chubb, 1980; 1982**). The parasites found in the cold freshwater fishes show seasonal fluctuations whereas, those found in warm freshwater fishes do not show any fluctuation with the season. Such variations have been correlated with the annual

temperature fluctuations of the water bodies (**Bauer and Karimov, 1990**). These authors reported that in cold-water bodies the temperature fluctuates throughout the year whereas, in warm water bodies the temperature remains constant during whole year. Similarly, **Brown (1989)** studied the effect of temperature on the prevalence and intensity of *P. laevis* in the fishes of controlled aquarium and suggested that temperature was the main factor in seasonal occurrence of this parasite. **Kennedy (1972a)** investigated the effect of temperature and other factors on the establishment and survival of *P. laevis* in experimentally infected *Carrassius auratus* and reported that temperature alone influenced the establishment of parasite. Low temperature (6°C) gave maximal establishment whereas, higher temperatures (12°C and 18°C) progressively reduced the establishment so that at 18°C the establishment was reduced by about 30% after one week. He further suggested that the reduced establishment of *P. laevis* during summer (high water temperature conditions) would be compensated by the increased feeding activity of the host so that the overall effect of parasite establishment does not change and worm population remains same throughout the year.

The intensity of *P. laevis* infection showed great variation in the range from 1-218 worms per host with mean intensity of about 34 worms (**Munro *et al.*, 1989**). **Dudiňák (2002)** studied the seasonal occurrence of *P. laevis* in the small Vihorlat Lake infecting the *Phoxinus phoxinus* as their definitive host and reported that there was no seasonal fluctuation in their occurrence and

the parasites were present throughout the year with the mean intensity of 6.6 worms per fish. **Brown (1986)** studied the density-dependent establishment and survival of ***P. laevis*** in the experimentally infected fish and reported that density-dependent establishment and survival occurred in such a number of parasites which survived to reproduce in an individual fish. He further observed a positive relationship between the fish length and worm burden of naturally infected *L. cephalus* with *P. laevis*.

Many workers reported the effect of seasons, sex and age of the host on the prevalence and maturation of *P. bulbocolli* **(Lawrence, 1970; Amin, 1987b)**. Maximum infection and maturation of the worms were reported during summer and minimum during autumn and female fish were more frequently and heavily infected than males but there was no relationship with the size of fish. **Kažič (1970)** reported the occurrence of *P. boniacus* from February to December with maximum infection in August. Similarly, **Amin (1975)** reported that the prevalence of *Acanthocephalus parksider* (North American species) also depended on host species, season of the year, size and sex of the host. The life cycle of parasite commenced in late summer/early autumn and reaches its peak during spring. **Muzzall (1980a)** studied the ecology and seasonal abundance of three acanthocephalan species like *P. bulbocolli, N. cristatus* and *Octospinifer macilentus* in freshwater fish *C. commersoni* (white suckers) in New Hampshire and reported that the prevalence of only *P. bulbocolli* fluctuates with the seasons. **Belghyti *et al.*, (1994)** studied the

population biology of *Acanthocephaloides propingues* infecting the *Citharus linguatula* and *Dicologoglosea cuneata* (Flat Fish) in the coastal water of Morocco, where 50% of fishes were found infected. They suggested that high prevalence of this parasite was due to high consumption of intermediate host (amphipod) and was not affected by the age of the fish. Concurrent infections with more than one species of acanthocephalan and /or with other helminths has been reported as a common phenomenon. *P. bulbocolli* and *Acanthocephalus dirus* occur concurrently in *Etheostoma caeruleum* where they occupy sites in close proximity to one another but no synergistic effect occurs **(McDonough and Gleason, 1981)**.

[II] Histopathological Studies:

Many species of parasites do cause considerable degree of alteration within their hosts and some of these may result in disease. Parasitic diseases caused by metazoans are usually a function of parasite density. Since, many species of zooparasites do not multiply within their hosts and therefore, the number of established parasites is equal to the number of parasites initially invading the host. Furthermore, the onset of recognizable disease symptoms are dependent upon a sufficient number of parasites present as well as the physiological state of the host **(Cheng, 1973)**. Thus, due to presence of small number of parasites in the host, no clinical symptoms appear. The damage caused by the parasite to their hosts and the tissue and humoral reaction of the

host against the parasite may be localized or spread all over the body depending upon the location of parasite on the host body.

Among helminths, only few species inhabiting the alimentary canal of fishes are pathogenic. Pathogenicity often results due to mechanical damage caused by the attachment organs with an inflammatory response at the site of attachment. The intensity of response is directly related to the depth to which the parasite penetrates. In addition to mechanical damage, the gut parasites also cause many functional disturbances in the host body including metabolic changes, retarded growth, weight loss, haematological changes and increased susceptibility to a variety of stresses resulting alterations in behaviour (reviewed by **Williams and Jones, 1994**).

Generally cestodes and acanthocephalans are firmly attached to the intestinal mucosa of the host with their scolex and proboscis, respectively. As a result different pathological events occur at the host-parasite interface. The pathological effects due to adult cestodes have been reviewed by **Rees** (1967). The mode of attachment of various cestodes like *Bothriocephalus scorpii*, *Clistobothrium crassieceps*, *Echinobothrium brachysoma* and *E. affine* has been investigated by **Rees (1958; 1961)**.

Many reports are available about the changes in the intestinal mucosa and underlying layers in response to parasitic infection which include displacement and flattening of the villi, mechanical injury, necrosis, loss of epithelia at or near the point of attachment, hyperplasia and metaplasia of the

mucosa, haemorrhage and destruction of mucosa with the host cellular reactions. The other histopathological changes related to the parasitic infection were reported as formation of lesions, ulcers, nodules, diverticula, calcareous, collagenous or fibrous capsules around the parasite, fibrosis and the appearance of granuloma (**Williams and Jones, 1994**). The destruction of intestinal villi, necrosis of the tissue and degenerative changes in mucosal epithelia adversely affect the absorptive efficiency of fish intestine thereby affecting the health and growth of fishes. **Castro *et al.*, (1967)** reported malabsorption in early infection of trichinosis and suggested that this was due to flattening of the mucosa and fusion of the villi.

The caryophyllidean cestodes include some highly pathogenic species, which cause a serious problem in farmed fishes. The degree of pathology in the gut is closely related to the morphology of the holdfast organs of the parasites. Those caryophyllidean species in which the specialized attachment organs are either absent (e.g. ***Atractolytocestus huronensis, Hunterella nodulosa, Khawia iowensis, C. laticeps***) or weak (e.g. ***Spartoides wardi, I. folous, G. catostomi, Biacetabulum biloculoides***) are more pathogenic because they burried their scoleces deep into the mucosa and thereby cause mechanical displacement and loss or compression of adjacent tissue. Among these species, ***H. nodulosa*** produce very large nodule with chronic inflammation, where the epithelia and lamina propria are lost and there is extensive lymphocyte invasion and hyperplasia of the submucosa. However, those caryophyllidean species

which have well developed /specialized but non-invasive attachment organs (e.g. *M. ingens*, *Capingens singularis*, *B. infreuens*, *B. caspiode*) are comparatively less pathogenic as they produce limited effect such as erosion of local epithelia without lesion or an inflammatory response (**Mackiewicz and McCrae,1962; Mackiewicz *et al.*, 1972; Hayunga, 1979**).

The caryophyllidean cestodes produce disease to the fishes by inducing mild irritation, inflammation between the folds, thinning of intestinal walls and sometimes death resulting from dysfunctioning of intestinal mucosa. The intestinal cells of the host become stretched and distorted causing mechanical obstruction of the lumen of fish intestine (**Bauer., 1968; Ahmad and Sanahullah, 1979; Scott and Grizzle, 1979**). During heavy infection, the intestine gets blocked causing death of the host (**Bauer *et al.*, 1981**). In some cases, high number of parasites reduce the diameter of the lumen by more than 50% which affects the movement of the food through the intestine (**Shostak and Dick, 1986**).

The other remarkable feature of the caryophyllidean cestodes is the presence of prominent secretory glands which are used by the parasites for establishment. The structure and function of scolex glands in different species of caryophyllidean cestodes were studied in detail by **Hayunga (1979)** and **Hayunga and Mackiewicz (1988)**. They reported that the scolex glands were more developed in those species, which lack attachment organs and suggested that the secretion of the glands was used by the parasite to adhere to the host

intestine. **Fotedar (1958)** reported well developed scolex glands in *A. orcini*, which extend to the posterior region in the form of three well developed columns. He suggested that the cestode attached to gut epithelia with the help of adhesive secretions of gland cells. The secretory glands in the scolex have also been reported in other groups of cestodes. **Farooqi (1958)** reported certain specialized cells in the rostellum of ***Taenia solium***. Small group of secretory cells located at the tip of rostellum of adult ***Echinococcus granulosus*** have been described by **Smyth (1963; 1964a)** who referred it as "rostellar gland". The secretory activity of these glands was demonstrated when 35 days old worms were incubated in warm saline. A drop of secretion was released which indicates that the rostellar gland becomes active as the worm progresses towards maturity **(Smyth, 1964a, Smyth *et al.*, 1969)**. The secretory activity of rostellar gland of ***E. granulosus*** at the host-parasite interface has been reported by using histological, histochemical and ultra-structural techniques **(Thompson *et al.*, 1979)**. It has been suggested that the secretion helps the parasite to attach itself at the host-parasite interface.

In contrast to caryophyllidean cestodes, the acanthocephalans have well developed proboscis with hooks for firm attachment with the intestine of the host. About one third species of acanthocephalans are found as adults in the intestine of fishes. The genus ***Pomphorhynchus*** has characteristic long hooked proboscis and bulbous neck, which are penetrated deep into the gut wall of their piscine host and lies permanently into the mucosal epithelium preferably

between the villi. As a result, a chain of dynamic interactions occurs at the host-parasite interface. Varying degrees of damage of host tissue and infiltration of cells have been reported in different fish species due to *Leptorhynchoides thecatus* and *P. bulbocolli* infections (**Venard and Warfel, 1953; Esch and Huffines, 1973; Hine and Kennedy, 1974; Dezfuli *et al.*, 1990**). **Wanstall *et al.*, (1986)** investigated the pathological alterations due to *P.laevis* infection in rainbow trout and reported that the presoma is penetrated into the mucosal epithelium, lamina propria, stratum compactum, stratum granulosum, muscularis and serosa of the gut. **Polzer and Taraschewski (1994)** reported that the cystacanths and adult *P. laevis* release proteolytic enzymes during *in vitro* incubation and concluded that the trypsin-like proteinase of both stages were necessary for the complete and quick perforation in the intestinal wall of fishes. Those acanthocephalan species which did not possess trypsin like enzyme needs more time to penetrate the intestinal wall than *P. laevis*.

Bullock (1963) made the histological observations of non-infected and infected intestines with *A. jacksoni* in some salmonid fish species like *S. salar*, *S. gairdeneri*, *S. trutta* and *Salvelinus frontinalis* and reported inflammation, destruction of epithelia and proliferation of connective tissue at the point of attachment in the intestine. **Chaicharn and Bullock (1967)** have reported the formation of a collagenous fibrous capsule around the proboscis of *P. bulbocolli* leading to the destruction of mucosa, stratum compactum and

muscularis layers at the site of attachment. Due to host's reaction, the parasite forms a fibrotic tunnel around its long slender neck and proboscis. **Dezfuli (1991)** described the detailed structure of the fibrotic tunnel. The tunnel terminates in a capsule, covered by serosa and mesenteries and protrude several millimeter into the coelomic cavity. Sometimes, these capsules persist as conspicuous fibrous nodules on the external surface of the alimentary canal or the proboscis perforates the capsule to emerge free in the coelom and penetrate other organs including liver. *A. anquillae* was reported to perforate the intestine of its host and attach to the liver **(Nickol, 1995).** Similar findings have also been reported for *P. bulbocolli* in *Etheostoma caeruleum* (Rainbow denters) by **McDonough and Gleason (1981)**. They further reported many histopathological changes like tissue hyperplasia and infiltration of leucocytes and eosinophils at the site of attachment. **Taraschewski (1989)** reported that large portion of liver was replaced by proliferative tissue often with patches of pancreas surrounding the embedded proboscis.

The foregoing literature survey clearly reveals that both caryophyllidean and acanthocephalan parasites cause considerable damage and therefore great economic losses to the fishermen. Thus, these groups of parasites require attention of parasitologists to develop an integrated control programme.

[III] Pathophysiological and Haematological Studies:

Various aspects of pathology due to adult cestodes have been investigated using only few species like *Hymenolepis diminuta, H. microstoma, Diphyllobothrium latum* and *Bothriocephalus* spp., and the literature on this aspect has been comprehensively reviewed by **Arme *et al.*, (1983). Lumsden and Karin (1970)** observed a typical inflammatory response with leucocytes invasion at the site of attachment of ***H. microstoma*** in rodents. Effects have also been reported from those organs which had no contact with the tapeworm. The cellular infiltration and fibrosis occurred in bile duct and later on lesions appeared on the liver surface **(Pappas, 1976)**. It has been suggested that the pathological changes like cellular infiltration and increased oxygen consumption in the organ not in contact with the parasites were initiated due to toxins produced by them **(Mayer and Pappas, 1976)**.

The parasites exert adverse effects on the host in different ways, for example, by feeding on blood, tissue destruction during larval migration, or mechanical or chemical irritation on contact surfaces, liberation of toxic metabolites, obstruction of excretory ducts and lumen of intestine, atrophy or hypertrophy of the tissue, etc. The structural alterations caused by the parasite result in the functional disturbances whose severity depends on location and degree of infection. Further, there is considerable variation in the ability of different organs to compensate the loss of functional tissue, for example the functional reserves in the liver are so large that dysfunction is evident only

after the loss of 60-70 % of the tissue (**Jubb and Kennedy, 1970**). The compensatory ability of the gastrointestinal tract was also reported in parasitic infection. **Symons *et al.*, (1971)** reported that the infected portion of the jejunum with ***Nippostrongylus brasiliensis*** show malabsorption, which was compensated by the adjoining uninfected area (ileum) of the intestine. On the other hand, organ systems with more limited functional reserve such as lungs have a lower tolerance level and dysfunction appears early.

The pathophysiological changes due to the parasitic infections have been comprehensively reviewed by many workers (**Soulsby, 1976; Neilson, 1982; Holmes, 1986; 1987**) but the pathophysiological changes due to parasitic infection among fishes have been poorly studied. The available literatures indicate that no work has been carried out on pathophysiology and haematology due to caryophyllidean cestodes and acanthocephalans infections in wild fishes; however, some reports are available on cultivated fishes. Reduction in growth rate, emaciation, loss of weight and appetite resulting in heavy mortalities have been reported among infected fishes with various cestodes and acanthocephalans (reviewed by **Williams and Jones, 1994**). Loss of total serum proteins and amino acids have been reported in *C. carpio* and *Clarias batrachus* infected with caryophyllidean cestodes (**Sapozhnikov, 1969; Kudryashova, 1970; Kadav and Agarwal, 1982; Kurovskaya, 1984**). *B. acheilognathi* and *K. sinensis* disrupt the intestinal and liver enzyme activity in *C. carpio* and thereby cause mortality of fishes (**Lozinska-Gabska,**

1981). It has been reported that caryophyllidean species *Capingentoides moghei*, causes loss of appetite, sluggishness and weight loss in *Heteropneustes fossilis* (**Jain *et al.*, 1976**). Further, decreased level of trypsin, chymotrypsin, alkaline phosphatase and increased level of acid phosphatase, amylases and proteases have been reported due to *B. acheilognathi* infection in *C. carpio* (**Matskasi, 1978; 1984; Sekretaryuk, 1983**). **Ranucci and Grol Ranucci (1978)** have recorded the changes in various serum enzymes including GOT and total and fractionated serum proteins, while no changes were found in GPT and alkaline phosphatase levels in sheep infected with hydatid cyst. **LeBars and Benting (1976)** reported significant changes in the level of various serum enzymes like ornithine carbamyl transferase (OCT), GOT and GPT during the course of development of *F. hepatica* in rabbits. Most of these studies have been carried out on experimental animals but an important question arises that whether these model animals can be used for pathophysiological studies. In this connection **LeBars and Benting (1976)** compared the pathological effect due to *F. hepatica* infection in sheep and rabbits and on the basis of differential response they suggested that rabbits couldn't be used as model for the study of pathological effects. **Symons (1976)** suggested that the most important pathophysiological response of the host to several gastrointestinal infections leads to anorexia exacerbated by the loss of serum proteins, which affect the protein metabolism.

Acanthocephalans were also reported to produce many pathological changes in the fish intestine (reviewed by **Nickol, 1995**). **Mann (1971)** has reported 17% weight loss of salmonid fishes infected with *N. rutili* and *Echinorhynchus trutta*. About 80% mortalities occur in *S. gairdneri* due to *E. salmonis* **(Bettocchi and Francálanci, 1963)**. Negative correlation has been demonstrated between the number of Acanthocephalans and body lipids and glycogen in *Salmo* species and *Gadus morhua* **(Bristol *et al.*, 1984; Buchmann, 1986)**. The trout infected with *P. laevis* had lower muscle protein than non-infected fish **(Wanstall *et al.*, 1982)**. They suggested that *P. laevis* induces mobilization of endogenous protein for energy production in preference to endogenous carbohydrates or lipids and reported that this is a common phenomenon in those fishes which are under stress. **Connors and Nickol (1991)** have reported significant detrimental effect on the flow of food energy due to *Plagiorhynchus cylindraceus* infection in fishes and thereby cause alteration in the basal metabolism.

Various haematological changes are known to occur due to various parasitic infections in sheep, cattle, rabbits and pigs **(Lean *et al.*, 1972; Kadhim, 1976; Maxie *et al.*, 1976; Holmes and Jennings, 1976; Jennings, 1976)**. A massive loss of RBC and plasma proteins due to helminth infections have also been reported **(Titchener *et al.*, 1974; 1975; Holmes, 1986; 1987)**. Such anaemic conditions generally explained when the packed cell volume or the haemoglobin concentration per unit volume falls below the accepted normal

range due to loss of blood. It has been reported that approximately 0.2 ml blood per worm per day was lost due to *Ancylostoma duodenale* and *A. caninum* infection in man and dogs, respectively (**Roche *et al.*, 1957; Miller, 1966**). The parasitic anaemia is caused either by haemorrhage or by direct consumption of blood, however there are some intestinal parasites where the mechanism of anaemia remains obscure. In such cases anaemia is not haemmorrhagic in aetiology and different causes have been attributed like digestive disturbances (**Gallagher, 1963**), inhibition of iron uptake (**Barker, 1973**) and due to toxins produced by the parasites which depress erythropoiesis (**Horak *et al.*, 1968**). Further, *D. latum* causes pernicious anaemia by utilizing vitamin B_{12} from the diet (see review by **Jennings, 1976**).

Reduction in haemoglobin level has been reported in *C. carpio* and *C. batrachus* infected with *K. sinensis* and *Lystocestus indicus*, respectively. *K. sinensis* reduced the number of erythrocytes, monocytes, polymorphs and neutrophills (See references in **Williams and Jones, 1994**). Similarly, **Sircar and Sinha (1974)** reported eosinophillia and macrocytic pernicious anaemia in fishes infected with *L. indicus*. Furthermore, decrease in haemoglobin, total blood volume, packed cell volume and elevated leucocytes, phagocytes and eosinophill counts and appearance of giant lymphocytes have been reported in *C. batrachus* and *C. carpio* due to helminthes infection (**Kudryashova, 1970; Kirichenko and Kosareva, 1972; Par, 1978; Svobodova, 1978; Kadav and Agarwal, 1983**). In addition to these changes, the physiology of the

gastrointestinal tract is also affected by parasitic infections (**Holmes, 1986**). The activity of brush border enzymes decreases due to parasitic infections and it has been suggested that it could be probably due to mucosal damage (**Jones, 1983**). In many infections, there are profound alterations in the intestinal absorptive surface, for example, in ascariasis, there is a shortening of intestinal villi and a loss of microvilli (**Stephenson *et al.*, 1980; Forsum *et al.*, 1981**). Atrophy of villi in the calves infected with *Trichostrongylus colubriformis* and *Nematodirus battus* has also been reported (**Coop *et al.*, 1973; Shayo and Benz, 1979**). In spite of structural damage, altered motility and secretions of the gastrointestinal tract, numerous attempts have also been made to determine whether impaired digestion and absorption are the major causes of poor utilization of food by parasitized ruminants. Although some studies suggest a reduction in the digestion or absorption of dietary nitrogen or other nutrients which are poor indicators of malabsorption (**Holmes, 1986**). Gastrointestinal leakage of plasma proteins particularly albumin is a well established fact in a number of infections of the small intestine (**Symons, 1982**). **Mulligan (1971)** suggested that loss of albumin into the lumen of large intestine occurs due to destruction of mucosa by the parasites. A general type of reaction due to gastrointestinal parasites is the production of inflammatory tissue and cells in the vicinity of the parasite with the loss of functional cells have been reported by **Neilson (1982)**. Hypertrophy of the tunica muscularis due to ascariasis in pigs leads to a considerable increase in gut weight (**Stephenson *et al.*, 1980**).

[IV] Biochemical Composition:

The survey of available literature reveals that very little work has been carried out on the biochemical composition of cestode parasites of fishes. Since, the biochemical composition depends on a number of factors, considerable variations exist between different species (**Smyth, 1969; Von Brand, 1979; Barrett, 1981; McManus and Bryant, 1986; Smyth and McManus, 1989**). Many complications arise in the study of cestode biochemistry due to the fact that various species like *E. granulosus*, *H. diminuta* and *T. crassiceps* have existed as complex of different strains which may often differ in their biochemistry and physiology. The biochemical composition may vary with respect to the strains of both parasite and host, age of infection, degree of maturation and nutritional status of the host (**Smyth and McManus, 1989**).

The major metabolic changes associated with the life cycle of the parasite are principally governed by various biotic and abiotic factors including catalytic potential and functions of enzymes and changes in their isozyme patterns (**Barrett, 1986**). This provides an opportunity to the parasites to switch on or off aerobic and anaerobic metabolism in response to environmental conditions. Such biochemical adaptations may be required for an immediate or long term metabolic needs of the parasites. **Roger and Petronijevic (1982)** suggested that the biochemical changes associated with the development of parasites are probably governed by a set of genes. These

genes may be stage-specific and require a trigger stimulus for activation or suppression. It is likely that a neurosecretory mechanism intervenes in the process, integrating the developmental changes with environmental cues. There is increasing evidence for the wide spread distribution of physiologically active neuropeptides in parasitic helminths (**Falkmer *et al.*, 1985; Wikgren *et al.*, 1986**), however, their involvement in the control mechanism has yet to be resolved.

Data on the biochemical analysis of cestodes are of limited value if the nutritional status of the host is not known, as significant fluctuations in individual parasite components can occur (**Smyth and McManus, 1989**). Many reports are available on the chemical composition of different species of cestodes infecting the fishes (**Jakurowicz and Korpaczewska, 1976; Strazhnik, 1980; Sterry and McManus, 1982**), but no information is available on *Adenoscolex*. In most of the adult tapeworms, the protein content was relatively low as compared to glycogen (**Smyth and McManus, 1989**). The total lipid content of helminths varied considerably and the value has been reported between 10-30% of the dry weight. The fatty acid composition of the cestode parasites depends on the diet of the host, as they are generally unable to synthesize it *de novo* (**Barrett, 1981**). Many physiological functions have been attributed to the lipids. It has been demonstrated by *in vivo* study that free sterols attract the worms whereas, phospholipids and other polar compounds act as a chemo-repellant (See review by **Haseeb and Fried, 1988**). Nucleic

acids normally constitute about 5-15% of dry weight of tissues and are involved in the storage, transmission and translation of genetic information **(Barrett, 1981)**.

As compared to cestodes, more information is available on acanthocephalans, in which the biochemical composition varied with the sex of the parasites **(Körting and Fairbairn, 1972)** and host **(Graff and Allen, 1963)**. Glycogen levels ranging from 0.6-2.4 g/100g wet weights have been reported from freshly isolated *Macracanthorhynchus hirudinaceus* females **(Donahue *et al.*, 1981)**. The glycogen levels equal to 22-24 % of the total dry mass have been reported from female *Moniliformis moniliformis* recovered from unstarved host **(Körting and Fairbairn, 1972; Starling and Fisher, 1978)**. **Dunagan (1964)** reported 20% glycogen of the tissue dry mass in *Neoechinorhynchus* species. Further, it has been reported that the glycogen content of acanthocephalans change dramatically with host feeding behaviour **(Crompton, 1970; 1972)**. The total lipid content was analyzed by **Vysotskaya and Sidorov, (1973)** in adult *E. salmonis* infecting freshwater fishes. These workers (loc cit) have reported more lipids in males as compared to females. Some acanthocephalan parasites of fishes are coloured due to the presence of different pigments. Adult *P. laevis* and *Filicollis anatis* contain unesterified β-carotene while adult *Rhadinorhynchus ornatus* and cystacanth of *Polymorphus minutus* contain only esterfied astaxanthin **(Barret and Butterworth, 1968; 1973)**. The exact function of these pigments in adult

acanthocephalans is not known but it has been suggested that the pigment of cystacanth (larval stage) may increase the probability of predation by the definitive host **(Starling, 1985). Barret and Butterworth (1968)** have suggested that these pigments are absorbed by the parasites from the host body.

[V] Protein Polymorphism:

The physiological significance and the ubiquitous distribution of proteins make them an integral component of every biological activity. Besides being building block, proteins are involved in the contractile system, transport mechanism, as protective agents, toxins, source of amino acids and energy fuel in absence of primary source. During cellular differentiation, the nucleo-cytoplasmic interaction causes qualitative and quantitative biochemical changes that ultimately lead to morphogenesis. The metabolic turnover accompanying the transformation from one stage to the next stage during the completion of life cycle involves qualitative as well as quantitative changes in various metabolites, which are greatly influenced by physico-chemical factors of different microhabitats.

Literature survey of the work done during the past several years clearly reveals that only a few species of helminth parasites, particularly ***Schistosomes, Fasciola, Hymenolepis*** and ***Echinococcus*** have been used for the study of biochemistry and protein synthesis along with immunological interactions. However, it is generally accepted that whenever the cellular differentiation and

asexual multiplication is involved, proteins and nucleic acids play a vital role. It has been observed in most of the invertebrates that the nucleic acid synthesis is followed by protein synthesis in cellular differentiation particularly, in gonadal development and maturation processes. Thus, it is evident that the protein synthesis is an important aspect in maintaining the parasitic mode of life. Hence, the study of protein polymorphism of parasites and immunological response of host may provide insight in the future prospects of the immunoprotection and immunodiagnostic studies.

The biochemical approach for the identification of organism found its way four decades ago when **Sibley (1960)** emphasized the application of electrophoresis as an analytical tool for solving the taxonomic problems. Since then, the electrophoretic separation of soluble proteins has been increasingly used in taxonomic studies of helminths **(Bylund and Djupsund, 1977; Le Riche and Sewell, 1978; Bullini, 1984; Bryant and Flockhart, 1986)**. Polyacrylamide has been widely used as a supporting medium for the electrophoretic separation of proteins for convenience in handling and better resolution.

Since, protein composition of an organism reflects the genetic constituents, therefore, it will provide a reliable measure of genetic differences between different organisms/strains **(Thompson and Lymbery, 1988; Lymbery and Thompson, 1988)**. Thus in the present study an attempt has

been made to analyze the soluble proteins of *Adenoscolex* and *Pomphorhynchus* isolated from fish by SDS-PAGE.

[VI] Immunological Studies:

The main role of immune system is to sustain the host defence mechanism and to maintain the homeostasis in the host. Analysis of the host immune response against the parasite has relevance in the diagnosis, protection and in understanding the immunobiology of the host against parasitic infection. It can be used for the evaluation of health status of the fish under different conditions, vaccination and breeding for disease resistance (reviewed by **Van Muiswinkel, 1995**). **Deplazes and Eckert (1996)** developed method for diagnosis of ***E. multilocularis*** infection in final host by ELISA and suggested that detection of circulating antibodies may be useful for primary screening of population. Fish are the oldest animal group with an immune system showing clear similarities with the defence system of mammals and birds. Both innate and acquired immunity exists in the fishes **(Hennessen, 1981; Van Muiswinkel and Cooper, 1982)**. The appearance of immunoglobulin (Ig) and other members of the 'Ig Super family' was observed for the first time in Pisces (reviewed by **Van Muiswinkel, 1995**).

The first line of defence in fishes includes structures which form stable physical and chemical barriers against invading pathogens. The physical barrier includes skin, gills and gut which prevent the entry of bacteria, fungi and

parasites (**Pickering and Richards, 1980**). The chemical factors include transferrin, lectins, C-reactive proteins, interferon, complements, etc which provide defence to the fish against various kinds of pathogens by different mechanisms (reviewed by **Van Muiswinkel, 1995; Buchmann *et al.*, 2001**). C- reactive protein has been detected in fish which binds with foreign materials (**White *et al.*, 1981**). **Fletcher (1982)** suggested that this protein might afford protection whilst specific antibodies are being synthesized.

The cell mediated immune response includes stimulation and transformation of lymphocytes, delayed hypersensitivity and mixed lymphocytic reaction (**Etlinger, 1975; Klein, 1977; Ellis, 1982**). **Ellis (1982)** concluded that the fishes lack typical histamine associated mast cells and IgE. Thus, they have alternate mechanisms for the production of inflammatory response than those demonstrated in higher vertebrates (**Davies and Lawson, 1985**). **Graves *et al.*, (1984)** reported the presence of non-specific cytotoxic cells in cat fishes but the exact biological function of these cells remain unclear. Acute inflammatory response and infiltration of granulocytes and macrophages have been observed in bony fishes, which increase with the severity of infection. (**Finn and Nielson, 1971**). **Warr and Marchalonis (1980)** concluded from occurrence and physiochemical properties of surface immunoglobulins that sub population of lymphocytes exists in teleosts. Surface immunoglobulins have been reported in 60-80% lymphocytes of spleen, thymus and peripheral blood of teleosts (**Warr *et al.*, 1977**).

The fishes so far investigated have only IgM, which is pentameric in chondrichthyes and tetrameric in teleosts (**Ambrosius *et al.*, 1982**), which is more suitable for agglutination and complement activation (**Alexander, 1982; Davies and Lawson, 1982**). Teleosts have effective IgM based humoral immunity which is not associated with class II region of the MHC (**Jurd, 1985**). **Kokuba *et al.*, (1987)** provided convincing evidence for the existence of IgM isotypes by molecular studies on the C-genes of shark. Like other vertebrates, the ability to phagocytose the foreign material appears in fishes during early development (**Finn, 1970; Grace *et al.*, 1981**). The non-specific immunity is soon supplemented by the rapidly developing cell-mediated components of immune system (**Manning *et al.*, 1982**). Similarly, the antibody response to certain antigens also matures quickly in fish (reviewed by **Mughal and Manning, 1995**).

It is evident from the available literature that the study of piscine immune response against helminth parasites is still in its infancy (reviewed by **Thomas and Woo, 1995; Dick and Choudhury, 1995**), however, more work has been carried out on protozoan, bacterial and viral infections. Several species of intestinal cestodes provoke non-specific type of immune response. **Kennedy and Walker (1969)** have reported by the experimental infection of ***C. laticeps*** in ***L. leuciscus*** that the parasites were initially established and then rejected by the host. On this basis they suggested the involvement of acquired immune response in parasite rejection. **Taylor and Hoole (1989)** reported

increase of melanomacrophages in the spleen of *Gobia gobia*, infected with *Ligula* spp., although cells count in pronephros remained unchanged. A delayed type of hypersensitivity and antibody response have been demonstrated against the antigen of young and adult *B. acheilognathi* in carp **(Kozinenko, 1981; Kozinenko and Balakhnin, 1981)**. A complement factor and C-reactive protein have also been implicated in leucocyte adherence to plerocercoid of *Ligula intestinalis* **(Hoole and Arme, 1986; 1988)**. It has been suggested that plerocercoid of *Ligula* and *Schistocephalus* adsorbed host proteins to evade host immune response. Specific antibodies have been detected in the sera of *A. brama* in response to *L. intestinalis* plerocercoid infection by Ouchterlony's double diffusion technique **(Berczi and Molnár, 1965; Molnár and Berczi, 1965)**, but **Sweeting (1977)**, using the same technique failed to detect precipitating antibodies in Roach. **Sharp *et al.*, (1989; 1992)** demonstrated the antibody response in the rainbow trout against *Diphyllobothrium* spp., by using ELISA.

Hamers *et al.*, (1992) reported interspecific differences in the leucocyte response in fishes parasitized by *Paratenisentis ambiguus*. In *A. anguilla* (the most suitable host of the parasite) the leucocyte response was much less intense as compared to *C. carpio* (Carp) and *O. mykiss* (rainbow trout), which are unsuitable hosts that expel the acanthocephalans within a few days. The leucocytes damage acanthocephalan tegument extensively in carp, hence they

speculated that cellular defence is a factor in determining host specificity of *P. ambiguus*. Precipitating antibodies to acanthocephalan antigens have been reported from sera **(Harris, 1970; Szalai *et al.*, 1988)** and intestinal mucus **(Harris, 1972)** of infected fishes and it was suggested that only mature worm produces antigenic substances. **Szalai *et al.*, (1988)** reported the presence of anti-Neoechinorhynchus carpiodi precipitins in serum of *Carpiodes cyprinus* and suggested that these precipitins were not complement reactive proteins or the alpha migrating factor. **Buchmann *et al.*, (2001)** reviewed the defence mechanism of fish against parasites and concluded that both non-specific and specific factors in the cellular and humoral branches of the fish immune system are well developed. Thus, production of specific antibodies in teleost B-lymphocytes against natural or synthetic antigens has been well characterized. MHC- class I and class II genes from a number of fishes have been sequenced which indicate that adaptive immune system in fishes is well developed (see references in **Buchmann *et al.*, 2001**). Many workers have shown the expression of a number of immune relevant genes in rainbow trout due to parasitic infection and suggested that the antibody production occurs both at a local and systemic level **(Lindenstrøm *et al.*, 2004; Sigh *et al.*, 2004)**

Recently **Chishti *et al.*, (2003)** reported the precipitating antibodies in *C. carpio* and *S. niger* against the soluble antigens of *A. oreini* and *P. kashmirensis* and suggested that the rate and magnitude of antibody production is a temperature dependant process. Similarly, it was suggested that

the immune response of *L. leuciscus* change with temperature which controls the incidence of *C. laticeps*, as it was demonstrated that this parasite established more successfully in their host at lower than higher temperature. **(Kennedy and Walker, 1969; Kennedy, 1969a; 1970).**

[VII] Pharmacological Studies:

Parasites have evolved many ways for surviving in nutritionally rich but immunologically hostile environments in their host. These adaptations make them unique and fascinating organisms to study. Parasites generally display a combination of biological and chemical adaptations unique in animal world and at the same time display a range of methods for evading the host immune response in order to establish a delicate and harmonious host-parasite relationship.

In view of the clinical and economic importance of the parasites, their eradication has received world wide attention. Control of parasites usually aims at reducing the parasitism to a level having no effect on productivity provided that it is economically feasible. Different methods of control adopted by parasitologists to achieve the goal include chemotherapeutic and immunological control measures. In the chemotherapeutic approach, a number of new compounds have been synthesized and screened for their efficacy against the parasites. The available literatures indicate that, in most of the cases

the mode of action is not completely known or if partially known, then their toxicological aspects have not been investigated.

Parasite chemotherapy has attracted considerable attention over the last two decades and many new drugs have been developed which possess greater degree of efficacy and safety than their predecessors. In 1909, **Paul Ehrlich** laid down the foundation of chemotherapy of parasites and proposed that the inhibition of enzymes that were crucial to parasites but not to the host, might be the basis of rational approach to chemotherapy of parasites. Since then, many chemicals have been screened for their chemotherapeutic effect and several reviews are available on this aspect (**Coles, 1983; Armour, 1983; Prichard, 1986; Bogan and Armour, 1986; Boray, 1986; Campbell, 1986; Campbell and Rew, 1986; Chappell, 1988**). A number of drugs belonging to the benzimidazole, salicylanilides, sulphonamides, halogenated phenols etc. are currently marketed which are safe, effective and broad spectrum anthelmintics, used against the common gastrointestinal nematodes of cattle, were also found effective against some cestodes and trematodes (**Van Den Bossche, 1980a, b; Schantz, 1982; Schantz *et al.*, 1982; Eckert, 1986;Ahmad *et al.*, 1987**). The drugs used for the treatment of tapeworm infections represent only few chemical classes and their pharmacology and mode of action have been reviewed by **Van Den Bossche (1985; 1986)**.

Numerous studies have been carried out to evaluate the effect of drugs on fish tapeworms. Several drugs have been found very effective against

Bothriocephalus and *Proteocephalus* spp. (reviewed by **Dick and Choudhury, 1995**). Niclosamide is the active ingredient present in the commercially available drugs (Devermin, Radeverm, Phenasal, Mansonil) and some medicated feeds (Zestocarp and Cyprinocestin) is suitable for dehelminthization of fishes. Synthetic anthelmintics, Dibutyl tin oxide were used successfully against cestodes and acanthocephalan parasites of fishes (see references in **Schäperclaus, 1991**). The anti-diarrhoeic drug Loperamide is effective in treating *S. gairdeneri* and other commercially cultivated fishes infected with *E. truttae* (**Taraschewski *et al.*, 1990**).

Different parameters have been used by various investigators for the assessment of the efficacy of drugs against the parasites. In recent years, the electron microscopy has been used as a powerful tool to study the actual site of drug action in the parasites. Since, the tegument of helminth parasites is metabolically active and plays an important role in transmembranosis of sugars, amino acids and other substances which are known to be influenced by various anthelmintics (**Van Den Bossche, 1972; 1976; Mansour, 1979; Chappel, 1988**) therefore, the tegument is being considered as site for drug action. Large numbers of reports are available on the *in vitro* effect of various anthelmintics on the tegument surface of cyclophyllidean cestodes and trematodes where different kinds of alterations have been reported (**Rogan and Richard, 1986; Ahmad *et al.*, 1987; Casado *et al.*, 1989**). However, very little information is available on the caryophyllidean cestodes and acanthocephalans.

Therefore, the topographical effect of various anthelmintics belonging to various chemical groups on the *Adenoscolex* and *Pomphorhynchus* has been studied. Further, the taxonomically different groups of parasites may respond differently to the same anthelmintics. Therefore, it is necessary to study each member of the drug separately in order to ensure disparity, if any, which might exist in the exact mode of action of drugs on different groups.

MATERIALS AND METHODS

MATERIALS AND METHODS

In order to find out the incidence of *A. oreini* and *P. kashmirensis* in the fishes of Kashmir, the Jehlum River was selected. This river originates from the Pir Panchal mountain range, few km south east of Verinag at 33° 30′ N latitude and 75° 25′ E longitude and flows in the north west direction of the valley covering three major districts of the Jammu and Kashmir State viz. Anantnag, Srinagar and Baramullah. The whole length of the river from its source to Baramullah is 241 km. In Anantang, six streams whereas, in Srinagar three large and many small streams and mountain torrents join the river (Fig. 5). Since, the river flows through a large area of the valley, it contributes to irrigation and is an important source of fish, water supply and provides recreation for the people. The highest pollution load to the river is found in Srinagar followed by Baramullah as indicated by physico-chemical parameters of pollution indicators (Fig. 6), which adversely affect the aquatic fauna of the river.

[I] Prevalence of *A. oreini* and *P. kashmirensis* in the fishes of Kashmir:

To collect the data on the incidence of these parasites in fishes, four economically important species of *Schizothorax* (*S. curvifrons*, *S. esocinus*, *S. labiatus* and *S. plagitomus*) were captured from four major sites with the help of local fishermen during the years 2000–2003. The collection of fish and thereby parasites were performed twice a week and data were recorded on

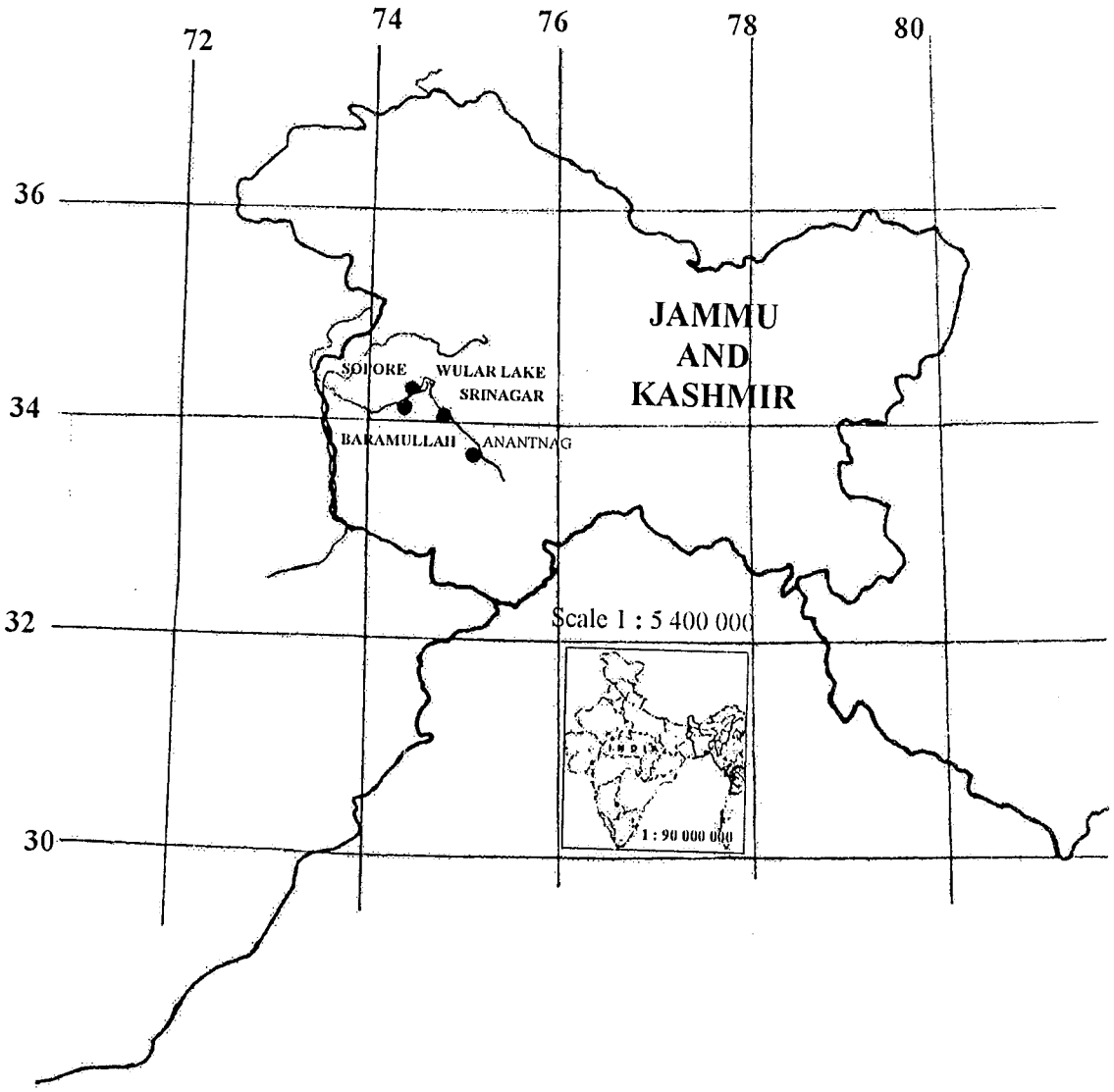


Fig. 5. Map of Jammu and Kashmir showing the collection sites of fishes from river Jehlum.

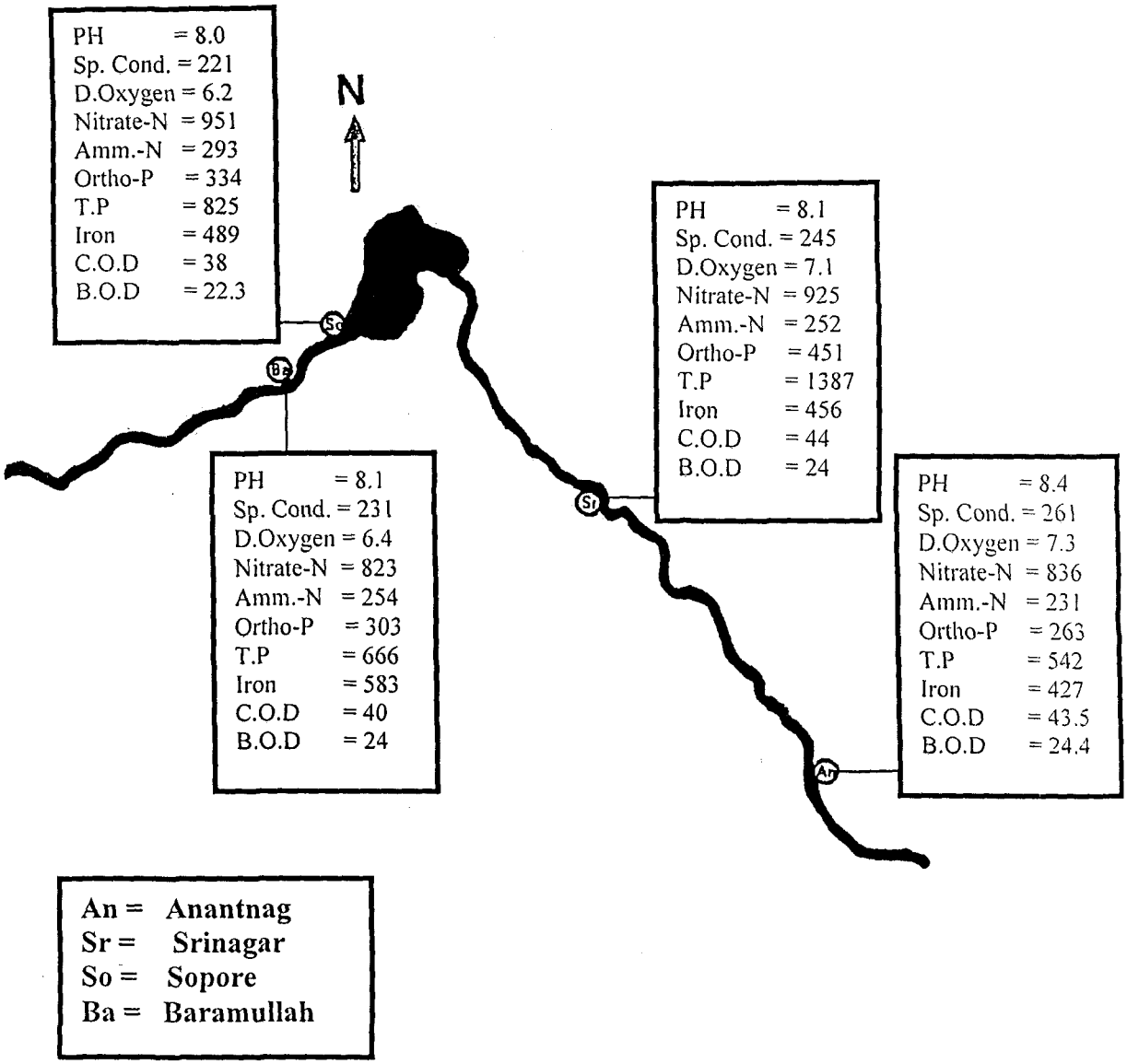


Fig. 6. Water quality of river Jehlum at different sites. [Source: Technical Report of J& K Lakes and Waterways Development Authority, 2000].

monthly basis from Anantnag, Sopore, Srinagar and Baramullah (Plate 3). It is worth mentioning that the fishes measuring 5cm or more were considered to find out the prevalence of these parasites. The juveniles and other fishes measuring less than 5 cm were not considered.

The fishes were brought alive to the laboratory and were kept in a well-aeriated aquarium. After recording the weight, length and sex of the captured fishes, they were autopsied and their intestines were examined for the presence of *A. oreini* and *P. kashmirensis* within 24 hours of collection. For isolation of worms, the intestines of infected fishes were incubated separately in the Hank's medium at 20–25°C. During the incubation, the cestode parasites detached from the intestine whereas, *Pomphorhynchus* were isolated with the help of forceps. After isolation, the total number of parasites per host was counted. The data were analyzed on the basis of body length and sexes of the fishes. Further, in order to find out the seasonal variations in the incidence of these parasites, the data were analyzed on the basis of months and seasons of the year.

[II] Histopathological Studies:

The intestine of naturally infected and non–infected fishes were fixed in Bouin's fixative and processed for various histopathological changes. After fixation, the materials were dehydrated through ascending grades of alcohol and cleared in xylene for about 30 min. Infiltration of paraffin wax in the tissue was carried out by giving two changes of 10 min each in melted wax at 56°C

Plate 3 **Collection sites of fishes in river Jehlum.**
(**An**=Anantnag, **Sr**=Srinagar, **So**= Sopore, **Ba**=Baramullah)

and then the materials were embedded in the paraffin wax. The sections of 5-7 μm thicknesses were cut on a rotary microtome and were floated on water drops on the albumin–coated slides, stretched by warming the slides on a stretching box. The slides were dried and used to observe the histopathological changes associated with the host tissue in eosin and haematoxylin stained sections.

[III] Pathophysiological Studies:

For pathophysiological studies, the blood samples were collected from naturally infected fishes during different months/seasons. The blood was taken directly from the heart using hypodermic syringe through the ventral side of the midline from the posterior margin of the operculum and directed dorso-caudally at an angle of 45° **(Lucky, 1977)**, stored in sterile vials with and without anticoagulants (EDTA) and kept at room temperature for 4 h for sera collection. After blood collection, the fishes were examined for the presence of parasites and on that basis the blood samples were separated as infected and non–infected. In the present study, only the blood of those infected fishes were analyzed which had only *A. oreini* or *P. kashmirensis* infection. However, the blood samples of those fishes having concurrent helminth infections were discarded and the protozoan and bacterial infections were ignored.

Sera samples were collected from the coagulated blood following centrifugation at 300 × g in a refrigerated centrifuge and stored at –20°C. The

sera of infected and non–infected fishes were used for the estimation of various enzymes, proteins (albumin and globulin), lipid and its fractions. The non-coagulated blood samples were used for the haematological studies. Besides this, the general body muscles and liver were isolated from infected and non-infected fishes and were used for the estimation of glycogen and proteins. The detailed procedure for extraction and estimation of these biochemical components is given in "biochemical composition of parasites" (Part V).

(a) **Transaminases:** Glutamate oxaloacetate (GOT, EC: 2.6.1.1) and glutamate pyruvate transaminase (GPT, EC: 2.6.1.2) were analyzed in the sera of infected and non–infected fishes. The activity of these enzymes was assayed spectrophotometrically at 340 nm using the diagnostic kit (Randox Laboratories Ltd., U.K.). These enzymes catalyze the transfer to α–ketoglutarate to yield L–glutamate and oxaloacetate or pyruvate. The reaction catalyzed by GOT is summarized below:

$$\alpha - \text{Oxoglutarate} + \text{L–Aspartate} \xrightarrow{\text{GOT}} \text{L} - \text{Glutamate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L} - \text{Malate} + \text{NAD}^+$$

For the assay of the activity of GOT, following reagents were used:

Contents	Concentrations in the test
1. Buffer/Substrate	
Tris buffer	80 m mol/l, pH 7.5
L–aspartate	240 m mol/l

2. Enzyme/Coenzyme/α–oxoglutarate

α–oxoglutarate	12 m mol/l
MDH	≥ 420 U/l
LDH	≥ 600 U/l
NADH	0.18 m mol/l

The assay mixture was prepared by adding equal volumes of buffer/substrate (1) and enzyme/coenzyme/α–oxoglutarate (2). From this mixture, 2 ml was pipetted in the cuvette to which 0.2 ml of the sera sample was added. The change of absorbance per min (ΔA) was recorded for 3 min at intervals of 1 min, and was used for the calculation of enzyme activity.

Enzyme activity (U/L) = 1746 × ΔA.

The activity of GPT was also assayed by spectrophotometric method. The reaction catalyzed by GPT is summarized below:

$$\textbf{α–Oxoglutarate + L–Alanine} \xrightarrow{\textbf{GPT}} \textbf{L–Glutamate + Pyruvate}$$

$$\textbf{Pyruvate + NADH + H}^{+} \xrightarrow{\textbf{LDH}} \textbf{L–Lactate + NAD}^{+}$$

In the assay, L–alanine was used in place of L–aspartate. The following reagents were used to find out the activity of GPT.

Contents	Concentrations in the test
1. Buffer/Substrate	
Tris buffer	100 m mol/l, pH 7.5
L–alanine	0.6 m mol/l

2. Enzyme/Coenzyme/α–oxoglutarate

α–oxoglutarate	15 m mol/l
LDH	≥ 1–2 U/l
NADH	0.18 m mol/l

Preparation of assay mixture and calculations of the enzyme activity was essentially the same as described above for GOT.

(b) **Acid (EC: 3.1.3.2) and Alkaline phosphatases (EC: 3.1.3.1):** These phosphatases were determined spectrophotometerically by using diagnostic kit (Randox Laboratories Ltd., U.K.). The reaction catalyzed by these enzymes is summarized below:

$$\text{p–Nitrophenyl phosphate} + H_2O \xrightarrow{\text{Phosphatases}} \text{p–Nitrophenol} + \text{Phosphate}$$

For the assay of acid phosphatase activity, following reagents were used :

Contents	Concentrations in the test
1. Buffer	
Citrate buffer	55 m mol/l, pH 4.8
2. Substrate	
p–nitrophenyl phosphate	5.5 m mol/l
3. Sodium tartrate	200 m mol/l
4. Sodium hydroxide	200 m mol/l

The assay mixture was prepared by adding equal volumes of substrate and citrate buffer (pH 4.8). From this solution, 1 ml was pipetted in the test tube and incubated for 5 min at 37°C. After 5 min incubation, 0.2 ml sera

sample was added and again incubated for 30 min. The reaction was stopped by adding total of 10 ml diluted sodium hydroxide at 30 second intervals. The change in absorbance of the sample was read against the reagent blank at 405 nm. The acid phosphatase activity was calculated by using the following formula

Total acid phosphatase (U/L) = 101 × ΔA

The alkaline phosphatase activity was determined using the following reagents:

Contents	Concentrations in the test
1. Buffer	
Diethanol amine buffer	1 mol/l, pH 9.8
$MgCl_2$	0.5 m mol/l
2. Substrate	
p–nitrophenyl phosphate	10 m mol/l

The assay mixture was prepared by adding equal volumes of buffer and substrate. From this solution, 3 ml was pipetted in the cuvette to which 0.05 ml of sera sample was added at 25°C. The contents were mixed and change of absorbance was recorded for 3 min at intervals of 1 min. The change in absorbance per min (ΔA) was used for the calculation of enzyme activity.

Enzyme activity (U/L) = 3300 × ΔA

(c) **Serum Lipids Profile:**

The total serum lipids were assayed using the diagnostic kit of Span Diagnostics Ltd., whereas, triglycerides and cholesterol were determined by using diagnostic kits of Crest Biosystems and Dr. Reddy Laboratories Diagnostic Division, respectively.

(i) Total serum lipids: The total serum lipid contents were determined by colorimetric method. The principle involved in its estimation is that lipids on heating with concentrated sulphuric acid and then mixed with phosphovanillin reagent produce pink coloured complex, which is measured by spectrophotometer (Spectronic 1001). For the assay, 0.05 ml of sera sample was taken in each test tube to which 2 ml of 36 N sulphuric acid was added. The standard lipids solution (700 mg %) was also run simultaneously which contain 0.05 ml standard lipids and 2 ml of 36 N sulphuric acid. The test tubes were mixed and kept in boiling water bath for 10 min. After cooling, 0.1 ml of sample was taken out from the above solutions (both experimental and standard) to which 3 ml of phosphovanillin reagent was added. The solution was mixed and incubated at 37°C for 15 min. The absorbance was recorded at 540 nm against the blank and the total serum lipids were calculated by using the following formula.

$$\textbf{Total serum lipids (mg/dl)} = \frac{\textbf{Absorbance test}}{\textbf{Absorbance std.}} \times 700$$

(ii) Serum Triglycerides: The principle of serum triglycerides estimation is based on the fact that the triglycerides are hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. The formed glycerol reacts with ATP in presence of glycerol– kinase to produce glycerol –3– phosphate which is oxidized by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxide further reacts with phenolic compound and 4–amino–anitipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. The intensity of colour formed is directly proportional to the amount of triglycerides present in the sample.

$$\text{Triglycerides} \xrightarrow{\text{Lipoprotein Lipase}} \text{Glycerol + Free Fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol –3–Phosphate + ADP}$$

$$\text{Glycerol– 3–Phosphate + } O_2 \xrightarrow{\text{Glycerol 3 PO}} \text{Dihydroxyacetone phosphate + } H_2O_2$$

$$H_2O_2 + \text{Aminoantipyrine + Phenol} \xrightarrow{\text{Peroxidase}} \text{Red Quinoneimine dye + } H_2O$$

For the assay of triglycerides, following reagents were used.

L_1: Enzyme reagent 1

L_2: Enzyme reagent 2

S: Triglycerides standard (200 mg/dl)

The working solution was prepared by mixing 20 ml of enzyme reagent 1 [L_1] with 5 ml of enzyme reagent 2 [L_2]. A total of 1 ml working solution was taken in each test tube to which 0.01 ml sera samples were added. Blank and standard were also run simultaneously, in which the sera sample was replaced

by the same amount of distilled water and triglycerides standard [S], respectively. The solution was mixed and incubated at 37°C for 5 min and the absorbance of standard and test sample was measured against blank at 505 nm. The quantity of serum triglycerides was calculated by using the following formula

$$\textbf{Serum triglycerides (mg/dl)} = \frac{\textbf{Absorbance test}}{\textbf{Absorbance std.}} \times 200$$

(iii) Serum cholesterol: Serum cholesterol estimation by Dr. Reddy's diagnostic kit is based on the modified methods of **Allain** ***et al.,*** (1974). Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol including those originally present, is then oxidized by the cholesterol oxidase to cholest–4–en–3–one and hydrogen peroxide. The hydrogen peroxide combines with phenol and 4–aminoantipyrine to form a chromophore (Quinoneimine dye), which can be quantitated at 500–550 nm.

HDL cholesterol fraction was also determined by precipitating other lipoproteins with precipitating reagent PEG 6000 and the supernatant was used to measure HDL cholesterol. Following reagents were used for the estimation of cholesterol and HDL cholesterol.

1. Buffer solution
2. Enzyme reagent
3. Cholesterol standard (200 mg %)

4. Precipitating reagent

5. HDL cholesterol standard (25 mg %)

The working enzyme reagent was prepared by mixing equal volumes of enzyme reagent (2) and buffer solution (1). For estimation of cholesterol, 1 ml working enzyme reagent was taken in each test tube including blank and test sample. In test sample 0.01 ml sera sample was added whereas, in blank 0.01 ml double distilled water was added in place of sera. The standard were also run simultaneously which had 1 ml working enzyme reagent and 0.01 ml standard cholesterol (3). All test tubes were incubated at 37°C for 5 min. The absorbance was read at 505 nm against blank. The amount of cholesterol was calculated by the following formula

$$\textbf{Total cholesterol (mg/100ml)} = \frac{\textbf{Absorbance test}}{\textbf{Absorbance std.}} \times 200$$

For HDL cholesterol assay, 0.2 ml of precipitating reagent (4) was added to 0.2 ml of sera sample and allowed to stand at room temperature for 5 min. The mixture was centrifuged at 3000 rpm for 10 min to obtain a clear supernatant. In assay mixture, 1.0 ml of working enzyme reagent was pipetted in the test tubes of blank, standard and test. In the test sample, 0.05 ml supernatant was added whereas, in blank and standard, 0.05 ml double distilled water and HDL cholesterol standard (5) was added, respectively in place of supernatant. These tubes were incubated at 37°C for 5 min. The colour was

read against blank at 505 nm. The HDL cholesterol was calculated using the following formula

$$\textbf{HDL cholesterol (mg/100ml)} = \frac{\textbf{Absorbance test}}{\textbf{Absorbance std.}} \times 50$$

The LDL cholesterol was estimated by using the empirical equation of **Friedewald *et al.*, (1972).**

$$\textbf{LDL cholesterol} = \textbf{Total cholesterol} - \textbf{HDL cholesterol} - \frac{\textbf{Triglycerides}}{5}$$

(d) Total Serum Proteins (Biuret method): Total serum proteins, albumin and globulins were also assayed in the sera of infected and non–infected fishes by using diagnostic kit (Ranbaxy Laboratories Limited). In alkali medium, the peptide bonds of proteins react with cupric ions in biuret reagent to form violet coloured complex. The intensity of colour depends on the concentration of total proteins in the sera. The biuret reagent contains 30 mM copper sulphate, 3.8 M sodium hydroxide and 100 mM potassium sodium tartrate. The working reagent was prepared by adding 1.7 ml copper sulphate (30mM), 0.7 ml sodium hydroxide (3.8M) and 0.6 ml potassium sodium tartrate (100mM). A total of 1 ml working reagent was pipetted in each test tubes including blank to which 20 µl sera was added. In the blank and standard 20 µl double distilled water and standard protein was added, respectively. All test tubes were shaken to mix the reagent and incubated at 25 °C for 20 min. The absorbance was read at 546 nm. The total proteins was calculated by the following formula

$$\text{Total Proteins (gm/ dl)} = \frac{A_T - A_B}{A_S - A_B} \times \text{Total protein concentration provided on calibrator label}$$

where, A_T = Absorbance of test
A_B = Absorbance of blank
A_S = Absorbance of standard

The globulin was separated from the sera by precipitating them with 35–40 % saturated ammonium sulphate and the albumin was estimated in the supernatant as described by **Dubey (1983)**. The concentration of albumin and globulin was determined by the biuret method as described above.

[IV] Haematological Studies:

Blood with anticoagulant collected from naturally infected and non-infected fishes during different seasons were used for various haematological investigations like erythrocyte and leucocytes count (TLC), differential leucocytes count (DLC), packed cell volume (PCV) and haemoglobin (Hb) content.

(a) Counting of total blood cells (RBC and WBC): The total blood cells count was carried out with the help of Neubauer haemocytometer (Hellige, West Germany). Thoma pipette was used for the blood dilution. In RBC counting the pipette was calibrated into 101 units while, for WBC it was calibrated up to mark 11. The blood samples were diluted in the ratio of 1: 200 with Hyem's fluid (containing mercuric chloride 0.25%, sodium chloride 0.5%

and sodium sulphate 2.5% in double distilled water) for RBC counting. However, for WBC, the blood samples were diluted in the ratio of 1: 20 with Dacie's fluid (containing 1% formaldehyde, 3.13 % of trisodium citrate and 0.1% brilliant cresyl blue) as described by **Blaxhall and Daisley (1973)**. The counting procedure of blood cells was essentially the same as described by **MacInnis and Voge (1970)**.

(b) **Differential Leucocyte Counts (DLC):** For differential leucocyte counts, thin blood smears were prepared on clean dry glass slides and fixed in methanol for 2 min. The blood smears were stained with Geimsa stain for 5 min and rinsed in tap water. The different cells were counted as described by **Ellis (1977)**.

(c) **Packed Cell Volume (PCV):** The packed cell volume was determined by the macro method as described by **Wintrobe (1967)**. The wintrobe tube was filled with blood and the level was adjusted to zero mark. Care was taken to avoid trapping of air bubbles during the filling of blood in the tubes. The tubes were then centrifuged at 3000 rpm for 15 min and the PCV was calculated by the following formula

$$\text{Packed cell volume} = \frac{\text{Height of packed cell column (mm)}}{\text{Total height of column (mm)}} \times 100$$

(d) **Total haemoglobin content:** The total percent haemoglobin (Hb) was estimated by using Sahli's haemometer with permanent coloured glass standards (Superior, Germany). The freshly collected blood of known volume was mixed with equal volume of 0.1N HCl in Sahli's haemometer. The haemoglobin content was determined by comparing the colour of the experimental solution with permanent coloured standard solution.

[V] Biochemical Composition of *Adenoscolex* and *Pomphorhynchus*:

A. oreini and *P. kashmirensis* were isolated from the infected fishes of Kashmir as described earlier. The isolated *A. oreini* and *P. kashmirensis* were pooled and washed several times in the Hank's balanced salt solution (pH 7.2). The parasites were weighed and used for estimation of various biochemical components.

(a) **Glycogen Assay:** The alkali soluble glycogen was extracted and estimated by the Anthrone Method of **Roe and Dailey (1966)**. For the extraction of glycogen, the materials of known wet weight were digested in 3ml of 1N NaOH in test tubes which were incubated in a boiling water bath for 30 min and shaken occasionally to facilitate the disintegration of tissue. After complete digestion, the tubes were cooled to room temperature and 6 ml of 1N perchloric acid was added and shaken vigorously and then allowed to stand for 15 min. The contents of the tube were centrifuged at 1500 ×g for 10 min and the precipitated protein was discarded. To the supernatant, 5ml of 95% ethyl

alcohol containing 0.1% lithium chloride was added and thoroughly mixed. The tubes were allowed to stand overnight at room temperature for glycogen precipitation and thereafter centrifuged at 1500 × g for 10 min. The supernatant was discarded and the glycogen pellet was washed twice with 95% ethanol containing 0.1% lithium chloride. Finally, the precipitated glycogen was allowed to stand for few hours for ethanol evaporation and then dissolved in known volume of double distilled water.

To estimate the glycogen content, suitable aliquots were added to 4 ml of Anthrone Reagent (containing 0.05% anthrone, 3% thiourea and 72% concentrated H_2SO_4 by volume). The tubes were then placed in a boiling water bath for 15 min and cooled before taking the absorbance against a reagent blank at 620 nm on spectrophotometer (Spectronic 1001, Bausch and Lomb, USA). Glycogen content was calculated with reference to a calibration curve, prepared by the known amount of standard glycogen (Sigma Chemical Company, USA).

(b) Protein Assay: Total protein content of the fresh homogenate was estimated by the dye binding method of **Spector (1978)**. Parasites of known wet weight were homogenized in 0.1N NaOH in a Teflon tissue homogenizer. After homogenization the debris was removed by centrifugation at 1000 x g and the supernatant was used for protein estimation. The dye binding reagent consists of Coomassie Brilliant Blue G–250 dye (0.01%), ortho–phosphoric acid (10%) and ethanol (5%). The protein content is measured linearly in the

range of 1.0 – 10 μg in 2.1 ml of the total assay volume at 595 nm. Bovine serum albumin (BSA) prepared in 0.1N NaOH was used as standard.

(c) Nucleic Acids Assay: For the extraction of RNA and DNA, known wet weight (0.5 g) of parasites was homogenized in 3 ml of 0.5N perchloric acid ($HClO_4$) and transfer to a stoppered test tube by washing with 1– 2 ml of 0.5 N $HClO_4$. The homogenates were heated in a water bath at 90°C for 20 min. After cooling, the mixtures were centrifuged at 500 ×g for 10 min and the supernatant was transferred to conical flask and the final volume made up to 10 ml with 0.5N $HClO_4$. This stock solution was used for the estimation of RNA and DNA.

(i) Ribonucleic Acid (RNA): The total RNA was estimated by the method of **Dische (1935)**. A total of 2 ml sample was taken from the stock and 4 ml freshly prepared orcinol reagent (containing 19.466 mg $FeCl_3$, 100 ml HCl and 3.5 ml of 6% orcinol in absolute ethanol) was added. The tubes were incubated at 100 °C for 5 min and after cooling, the colour was read at 665 nm against the reagent blank. The total RNA content was calculated from a previously prepared standard curve using purified RNA (Sigma Chemical Co., USA).

(ii) Deoxyribonucleic acid (DNA): Total DNA was estimated by the method of **Giles and Myers (1965)**. Aliquots of 2 ml samples were taken in stoppered tubes in which 4 ml Burton's diphenylamine reagent (containing 1.5 g diphenylamine in 100 ml glacial acetic acid and 0.5 ml of 1.6% acetaldehyde solution) was added. The tubes were incubated overnight at 30 °C for 12–16 h.

The colour was read against the reagent blank at 600 nm. A standard curve was prepared for the calculation of total DNA content using purified DNA (Sigma Chemical Co., USA).

(d) Assay of Total Lipids: The total lipid contents were extracted by the method of **Folch *et al.*, (1957)** as modified by **Misra (1968)**. The parasite materials of known wet weight were kept overnight in 20 fold volume of chloroform:methanol (2:1 v/v), following which the parasites were homogenized in the same solvent medium. The residue was then removed by centrifugation at 400 ×g for 5 min which was again re–extracted with chloroform:methanol (2:1) and both the supernatant were combined. To the chloroform : methanol extract, 0.2 ml of 0.88% KCl was added , mixed and allowed to separate into two phases. The lower phase was evaporated to dryness *in vacuo* at 45 °C. The dried lipids were then dissolved in known volumes of chloroform and suitable aliquots were taken for the estimation of total lipids.

The total extracted lipids were estimated by the method of **Zöllner and Krisch (1962)**. To 1 ml of extracted lipids, 4 ml of concentrated H_2SO_4 was added. After shaking, the tubes were boiled for 10 min then cooled to room temperature. Suitable aliquots were taken and 4 ml of Zöllner reagent (containing 13 mM vanillin in 14 M ortho–phosphoric acid) was added. A reagent blank was also prepared simultaneously by using H_2SO_4 and Zöllner reagent. Both test and blank tubes were kept at room temperature for 30 min.

The colour was read against the reagent blank at 530 nm. The total lipids were calculated from a previously prepared calibration curve using a standard lipid of known concentration.

[VI] Electrophoretic Analysis of Soluble Proteins:

In order to study the protein polymorphism, the fish parasites *Adenoscolex* and *Pomphorhynchus* were washed and homogenized in 0.1M phosphate buffer (pH 7.4) containing 0.25M sucrose in a glass Teflon tissue homogenizer with a motor driven pestle. The homogenates were centrifuged at 1000 ×g for 10 min to remove debris and unbroken cells. Protein concentration of the samples was determined by the method of **Spector (1978)**.

(a) SDS Polyacrylamide Gel Electrophoresis (SDS–PAGE): Polypeptide profile was analyzed by SDS–PAGE using discontinuous buffer system as described by **Laemmli (1970)** with some minor modifications. The separating and stacking gels were prepared from the various stock solutions.

(i) Acrylamide Solution:

Acrylamide	30.0 % (w/v)
Methylene–bis–acrylamide	0.80 % (w/v)

(ii) Separating Gel Buffer:

Tris	36.30 g
Temed	0.23 ml
SDS	0.80 g

Dissolve in 80 ml of DDW, adjusted to pH 8.9 with 1N HCl and made to 100 ml with DDW.

(iii) Stacking Gel Buffer:

Tris	5.98 g
Temed	0.46 ml
SDS	0.80 g

Dissolve in 80 ml of DDW, adjusted to pH 6.8 with 1N HCl and made to 100 ml with DDW.

(iv) Running Buffer:

Tris	9.09 g
Glycine	43.20 g
SDS	3.00 g

Dissolve in DDW, adjusted to pH 8.3 with 1N HCl and made to 1000 ml with DDW. For use, diluted to 1:2 with DDW.

(v) Laemmli's Sample Buffer:

Tris	0.968 g
SDS	2.0 g
β– Mercaptoethanol	5.0 ml
Glycerol	10% (v/v)

Dissolved in DDW, adjusted to pH 6.8 and made to 100 ml with DDW.

(vi) Ammonium Persulphte (APS):

10% (w/v) freshly prepared solution in DDW was used.

Gel Preparation: The separating gel of 10% (homogenous gel) was prepared by mixing 10.7 ml of acrylamide solution, 5.0 ml of separating gel buffer and 16.3 ml DDW and degassed for 10 min at 25 lb negative pressure. After

degassing, freshly prepared 10% ammonium per sulphate was added. The gel mixture was immediately poured into the glass plates mould (17 × 17 cm size) using 0.1 cm thick spacers. Once the gel solution was poured, it was carefully overlaid with a few drops of DDW and allowed to polymerise at room temperature for 45 min. After polymerization, distilled water was carefully removed from the gel surface and then 4% stacking gel solution (containing 1.33 ml acrylamide, 1.33 ml stacking gel buffer, 150 µl of APS and 7.18 ml of DDW) was poured onto the separating gel. Simultaneously desired combs were inserted and gel solution was overlaid with few drops of DDW. The gel solution was allowed to polymerize at room temperature for 30 min.

Sample preparation: The protein sample (concentration pre–maintained to 2 µg/µl with desired buffer) was mixed with equal volume of Laemmli's sample buffer. Aqueous bromophenol blue (0.05%) was used as marker dye. The sample mixture was boiled for 8 min at 100°C in boiling water bath. The standard molecular weight markers were also run simultaneously. The low molecular weight standard protein markers purchased from Genei contained phosphorylase b (97.4 KD), bovine Serum albumin (68 KD), ovalbumin (43 KD), carbonic anhydrase (29 KD), soyabean trypsin inhibitor (20 KD) and lysozyme (14.3KD). The protein samples along with standard markers were carefully loaded onto the gel with the help of micro sample applicator.

Electrophoresis: Electrophoresis was carried out at 12 mA/slab gel in vertical slab gel system (Biotech R & D Laboratories, India). The temperature of the

system was maintained at 6 °C. When the bromophenol blue tracking dye reached 0.5 cm above the end of the gel, power supply was disconnected and gels were removed from the glass mould and used for staining.

Staining of Gel:

(i) Coomassie Brilliant Blue (CBB R–250) Staining: After electrophoresis, gels were fixed in fixing solution consisting of 10% (v/v) acetic acid, 45% (v/v) methanol, for 2 h. Thereafter, gels were stained with 0.25% (w/v) CBB R–250, prepared in fixing solution, overnight. The over stained gels were destained in high destaining solution, consisting of 7% (v/v) acetic acid and 45% (v/v) methanol. Finally gels were destained in low destaining solution consisting of 7% (v/v) acetic acid and 5% (v/v) methanol in DDW, until the background became clear. Finally gels were stored in 7% (v/v) acetic acid in DDW.

(ii) Silver staining: Silver staining of gels was preformed following the method of **Oakley *et al.*, (1980)** with some modifications which is about 10 times more sensitive than coomassie staining. All the procedures were performed at room temperature with continuous gentle shaking and lighting. Double deionized glass distilled water was used to prepare all the solution, and handling of gels was minimized by using an aspirator to remove reagents and washing solutions. All staining procedures were carried out in (34 × 20 × 5.6 cm) glass dishes. The gels were prefixed with 12.5 % (w/v) trichloroacetic acid in 65% (v/v) methanol for 1h and then fixed in 10% unbuffered glutaraldehyde prepared by diluting 25% analytical grade glutaraldehyde for 30 min. The gels

were then rinsed in DDW for 6 h with several changes, and transferred to freshly prepared dithiothreitol solution (5 mg/l DDW) for 35 min. Thereafter, the gels were incubated in silver nitrate solution (0.1% w/v $AgNO_3$ in DDW) for 40 min. After rinsing, gels were transferred to freshly prepared developer solution (3% sodium carbonate containing 0.05% v/v of 37 % formaldehyde) for 3–5 min. As soon as transient brown colour developed, solution was decanted and fresh developer was added until the appropriate colour developed. The reaction was stopped by adding 5 ml of 2.3 M citric acid per 100 ml of developer. After washing with DDW for about 30 min, the gels were stored in 0.03% (w/v) sodium carbonate solution. The stained gels were photographed under transillumination by Canon AEI camera using Kodak Max 400 colour film.

(iii) Molecular Weight Determination: The relative mobility of each polypeptides were calculated by the following formula

$$\mathbf{Rf} = \frac{\textbf{Distance of polypeptid es migrated}}{\textbf{Distance of tracking dye migrated}}$$

The Rf value of individual polypeptide was calculated with the help of their migration distance. Subsequently molecular weight (Mr) of each polypeptide was determined with the help of semi log standard graph plotted by known molecular weight standard marker proteins against their Rf values (Fig. 7).

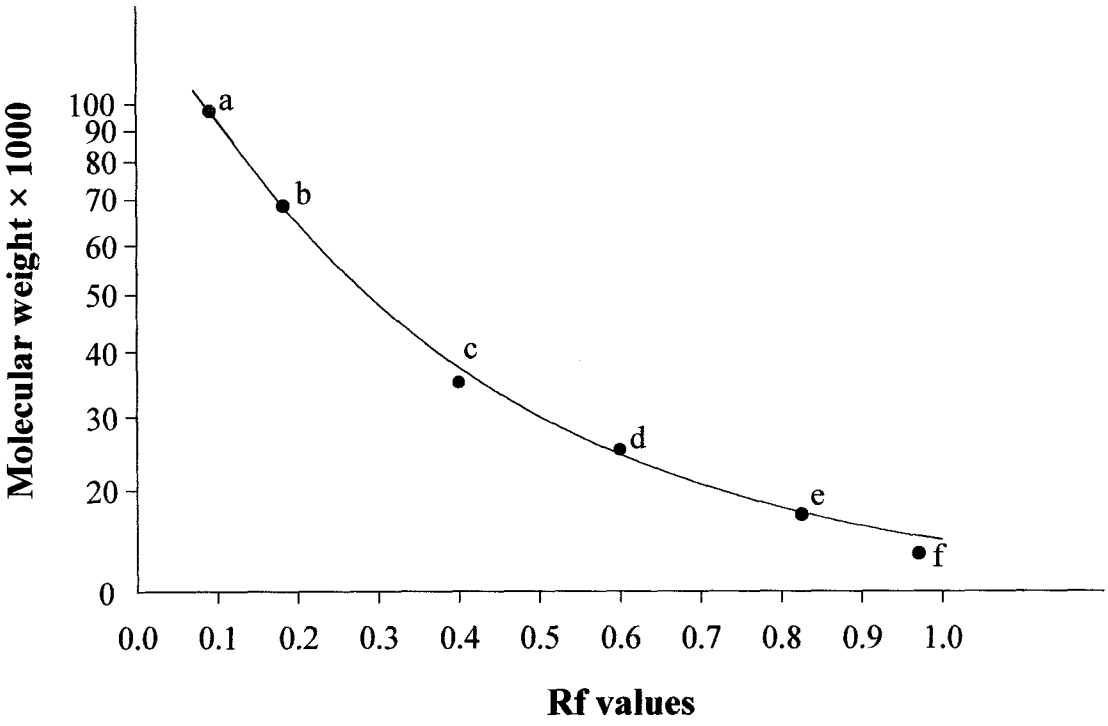


Figure 7. Calibration curve of standard molecular weight marker proteins in KD.

a. Phosphorylase b	97.4
b. Bovine serum albumin	68.0
c. Ovalbumin	43.0
d. Carbonic anhydrase	29.0
e. Soyabean trypsin inhibitor	20.0
f. Lysozyme	14.3

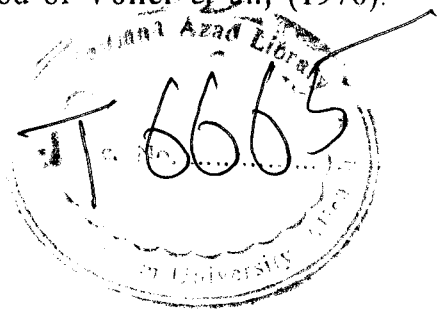
[VII] Immunological Studies:

For immunological studies, *A. oreini* and *P. kashmirensis* were homogenized separately in 0.1M PBS (pH 7.3) in Teflon tissue homogenizer. After centrifugation at 5000 ×g, the supernatant was separated and stored at –20 °C. The amount of protein was calculated using the dye binding method of **Spector (1978)**. The soluble homogenates of the parasites were inoculated along with Freund's complete adjuvant in the experimental animals (rabbits), separately. Before inoculation of antigen, the blood from marginal ear vein was collected and used as control. In I^{st}, dose 500 µg antigen was inoculated and thereafter, I^{st}, II^{nd}, III^{rd} and IV^{th} boosters were given at 5 days intervals having antigen concentrations as 500 µg, 400 µg, 300 µg and 250 µg, respectively. After 4 days of last booster, the blood was collected from marginal ear vein in sterile vials without anticoagulant and allowed to stand at room temperature for 4 h. The sera samples were collected from coagulated blood, centrifuged at 3000 × g and stored at –20 °C. These sera samples were used for the immunological studies. The blood samples were also collected from naturally infected fishes with *A. oreini* and *P. kashmirensis* separately, during the month of April. The blood of those fishes which had mixed infection or any other infection was discarded. The sera samples were collected as described above.

(a) Ouchterlony's Double Diffusion Test: Ouchterlony's Double Diffusion test was performed as described by **Ouchterlony (1948)**. 1% agarose gel was prepared in 0.1M phosphate buffer (pH 7.4), and about 5 ml was poured on pre

cleaned glass slides. After 10–15 min, the wells were punched and sealed with 0.3% agarose. The slides were kept in humid chamber and the wells were filled with antigens and raised antisera and left overnight. Controls were also run simultaneously which contain non-immunized / non-infected sera and antigens of *A. oreini* and ***P. kashmirensis***. After completion of diffusion, the slides were removed from humid chamber, washed several times in normal saline and then in double distilled water and kept in thermostat at 40 °C for 12 h. The slides were stained in CBB R–250 for 30 min and destained in destaining solution containing methanol, acetic acid and double distilled water in the ratio of 4:1:4.

(b) Enzyme–Linked Immunosorbant Assay (ELISA): To monitor the antibody titre, ELISA was preformed by the method of **Voller *et al.*, (1976)**. The following buffer/solutions were prepared.



(i) Coating Buffer (pH – 9.7):

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
DDW	1000 ml

(ii) Washing Buffer: Phosphate buffered saline–Tween (PBS–Tween)

Na_2PO_4	5.80 g
KH_2PO_4	1.63 g
NaCl	5.00 g
Tween–20	0.50 ml
DDW	1000 ml

(iii) Diethanol Amine Buffer (DEA buffer):

Diethanol amine	48.5 ml
NaN_3	0.1 g
$MgCl_2$	50 mg

The final volume was adjusted to 500 ml with DDW.

To each well of a microtitre plate (Dynatech, USA), 50 µl of antigen solution in coating buffer with a protein concentration of 10 µg / ml was dispensed and incubated at 37 °C until it was air-dried. The plates were washed by filling and emptying and refilling the wells with washing buffer for 3 × 10 min. The unwanted reactive sites were blocked by filling each well of the microtitre plate with 150 µl of 5% low fat milk (Nestle, India) in phosphate buffered saline and incubated at room temperature for 3 h. The plates were again washed with washing buffer for 3 × 10 min. The test sera collected from the control and experimental animals were diluted serially in PBS containing 0.5% milk protein. About 50 µl of these serially diluted sera were loaded in each well of the plates, and then incubated for 3 h at room temperature in a humid chamber. The plates were washed for 3 × 10 min with the washing buffer. Thereafter, in each well of the 96 wells plate, 50 µl of secondary antibody conjugated with alkaline phosphatase (anti rabbit IgG developed in goat) at a dilution of 1 : 2000 was added and the plates were incubated for 3 h at room temperature in a humid chamber. After incubation, the plates were washed for 3 × 10 min with washing buffer. The colour was developed by

adding 100 µl of substrate p–nitro phenyl phosphate (Sigma Chemical Companies, USA) at a concentration of 1mg per ml in 10% DEA buffer. Finally the reaction was stopped by adding 50 µl of 3N NaOH solution to each well. The plates were read out at 405 nm in an ELISA reader (SLT Lab. Instrument, Austria).

[VIII] Topographical Effect of Drugs:

In order to observe the effect of various known anthelmintics, ***Adenoscolex and Pomphorhynchus*** were incubated with drugs under *in vitro* conditions and the effect was monitored by Scanning Electron Microscope (SEM). The solvent of drugs and their concentrations are summarized in Table 1.

Among various drugs, mebendazole was dissolved in dimethyl sulphoxide (DMSO) which has been reported to be a safe drug solvent for helminthes **(Ahmad and Nizami, 1983)**. Besides this, other drugs like rafoxanide, nitroxynil and clorsulon were dissolved in ethanol and monensin in double distilled water. Care was taken that the solvent concentration would remain constant as 3% ethanol and 0.1% DMSO as the case may be in the final incubation mixture. In every experiment, suitable controls were run simultaneously.

Freshly isolated ***A. oreini*** and ***P. kashmirensis*** were washed and pre-incubated in HBSS (without glucose) for 1 h at 27 ± 2 °C. After pre-incubation,

Table 1. Drug concentration and their solvents.

Group of the drugs	Drugs used	Solvent medium	Drug concentrations (μ mol per ml of incubation medium)		
Benzimidazole	Mebendazole	DMSO	33.0×10^{-2}	66.0×10^{-2}	99.0×10^{-2}
Salicylanilide	Rafoxanide	Ethanol	2.03×10^{-2}	4.06×10^{-2}	6.09×10^{-2}
Halogenated Phenols	Nitroxynil	Ethanol	1.72×10^{-2}	3.44×10^{-2}	5.17×10^{-2}
Sulphonamide	Clorsulon	Ethanol	1.32×10^{-2}	2.64×10^{-2}	3.96×10^{-2}
Ionophores	Monensin	Water	0.72×10^{-2}	1.44×10^{-2}	2.16×10^{-2}

DMSO – Dimethyl Sulphoxide

the worms were washed in two changes of HBSS (without glucose) and then incubated in HBSS (with glucose) with and without drugs for 3 h at 27 ± 2 °C. Controls were also run simultaneously in HBSS containing only drug solvents. After incubation with drugs and drug solvents, the parasites were isolated, washed gently with HBSS and fixed as described by **Irshadullah *et al.*, (1990)**. The parasites were fixed in 4% buffered glutaraldehyde (pH 7.2) at 4 °C overnight. Subsequently, they were washed in cold 0.1M cacodylate buffer (pH 7.4) without sucrose, giving 3 × 80 min changes and were post fixed in 1% osmium tetra oxide in Millonig's buffer (pH 7.2) for 3 h at 4 °C and then transferred directly to 50% ethanol. After dehydration in graded series of ethanol, the specimens were transferred to 100% acetone and critically dried using liquid CO_2 as the transitional fluid. The worms were then mounted on the aluminium stubs using silver epoxy and coated with gold by using Hitachi (HUS–5) vacuum evaporator coating unit. The specimens were viewed at different resolutions using Hitachi (S 3000–H) scanning electron microscope, operating at 5/30 KV.

RESULTS

RESULTS

[I] Prevalence of *A. oreini* and *P. kashmirensis* in the fishes of Kashmir:

In order to investigate the prevalence of *A. oreini* and *P. kashmirensis* in the fishes of Kashmir, a survey was carried out during 2000–2003 at four different sites (Anantnag, Srinagar, Sopore and Baramullah) of river Jehlum and four species of *Schizothorax* were examined. A total 28.44 % fishes were found infected with these parasites among which, 12.27 % fishes harboured *A. oreini*, 12.07 % *P. kashmirensis* and 4.1 % both *A. oreini* and *P. kashmirensis* infection. The intensity of infection varied with the parasite species and maximum number of parasites per host was observed for *A. oreini* (Table 2). It was further noticed, that the incidence of these parasites varied with the site of collection. Maximum fishes were found infected with *A. oreini* in Srinagar and *P. kashmirensis* in Sopore (Table 3, Fig. 8). All four species of *Schizothorax* were found infected with *A. oreini* and *P. kashmirensis* and considerable variations were noticed in the prevalence rate of these parasites. Among these species, *S. esocinus* was found more infected than any other species (Table 4). The prevalence of *A. oreini* was slightly more in *S. plagitomus* followed by *S. esocinus* whereas, *P. kashmirensis* infection was found maximum in *S. esocinus* followed by *S. labiatus* (Fig. 9).

Marked variations were noticed in the rate of infection during various seasons. Maximum fishes were found infected during spring followed by winter (Table 5) but the infection of individual parasites showed a different

Table 2. Prevalence of *A. oreini* and *P. kashmirensis* in *Schizothorax* species collected from Kashmir Valley.

Parasite Species	No. of fish infected	Percent Prevalence	No. of parasites obtained	Intensity of infection
Adenoscolex oreini	123	12.27	3259	26.49
Pomphorhynchus kashmirensis	121	12.07	1589	13.13
Both *A. oreini* and *P. kashmirensis*	41	4.10	564	13.80
Total	285	28.44	5412	18.90

No. of fish Examined = 1002

Table 3. Prevalence of *A. oreini* and *P. kashmirensis* in *Schizothorax* species collected from different sites of Kashmir valley.

Sites of collection	Total no. of fish examined	No. of fish infected with *A. oreini*	No. of fish infected with *P. kashmirensis*	No. of fish infected with both *A. oreini* and *P. kashmirensis*	Total percent prevalence
Anantnag	210	18 (8.57%)	21 (10.0%)	5 (2.38%)	20.95
Srinagar	237	53 (22.36%)	31 (13.0%)	10 (4.22%)	39.58
Sopore	358	40 (11.17%)	54 (15.08%)	19 (5.31%)	31.56
Baramullah	156	12 (7.69%)	15 (9.62%)	7 (4.49%)	21.80

Values in parentheses show the percent prevalence.

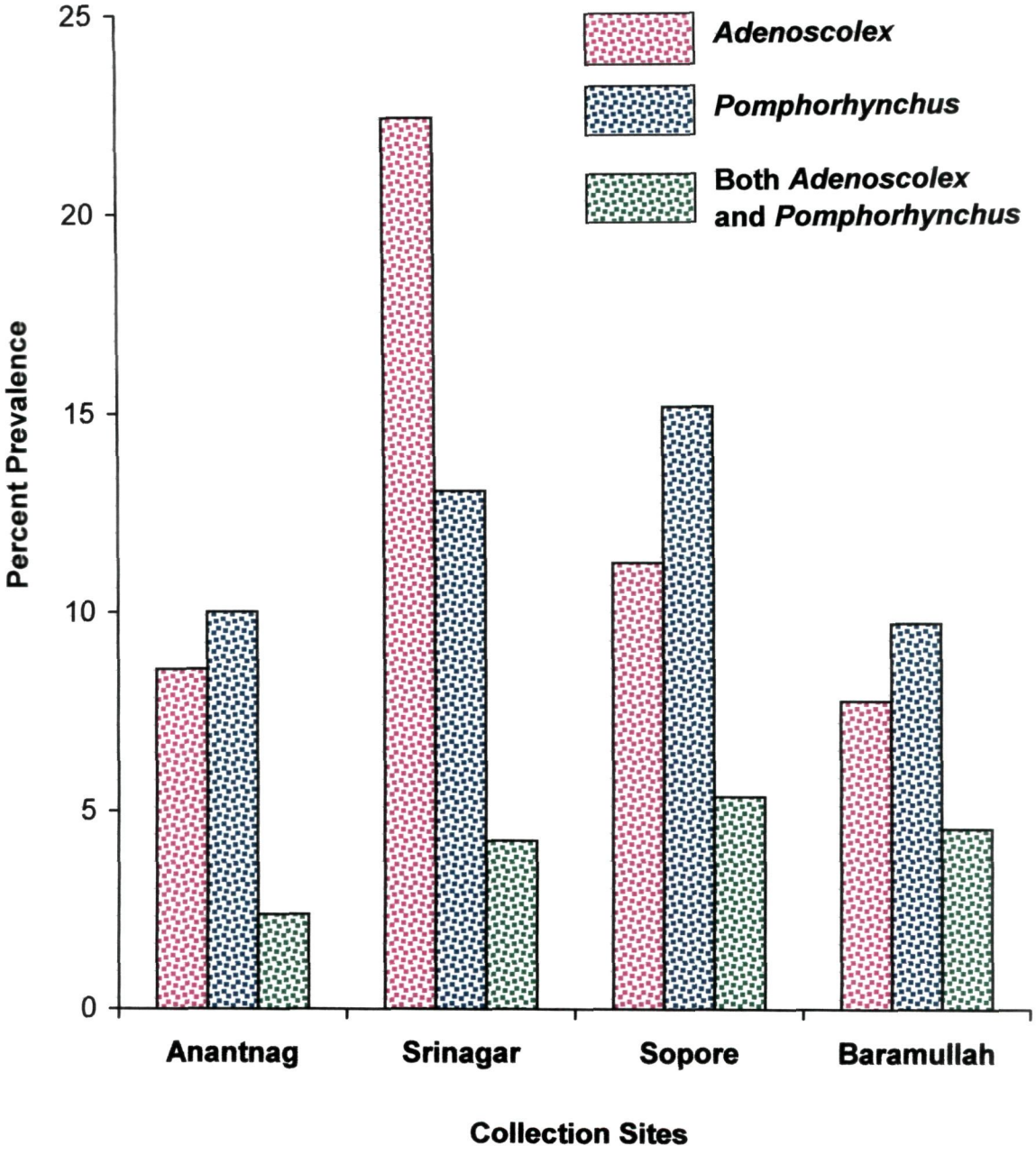


Fig. 8. Percent prevalence of *A. oreini* and *P. kashmirensis* in *Schizothorax* species collected from different sites of Kashmir Valley.

Table 4. Prevalence of *A. oreini* and *P. kashmirensis* in four major *Schizothorax* species of Kashmir Valley.

Fish species	Total no. of fish examined	Total no. of fish infected	No. of fish infected with *A. oreini*	No. of fish infected with *P. kashmirensis*	No. of fish infected with both *A. oreini* and *P. kashmirensis*
S.curvifrons	320	79 (24.7%)	39 (12.2%)	28 (8.6%)	12 (3.6%)
S.esocinus	294	100 (34.0%)	37 (12.6%)	49 (16.7%)	14 (4.8%)
S.labiatus	175	49 (28.0%)	19 (10.9%)	23 (13.1%)	7 (4.0%)
S.plagitomus	213	57 (26.8%)	28 (13.1%)	21 (9.9%)	8 (3.6%)

Values in parentheses show the percent prevalence.

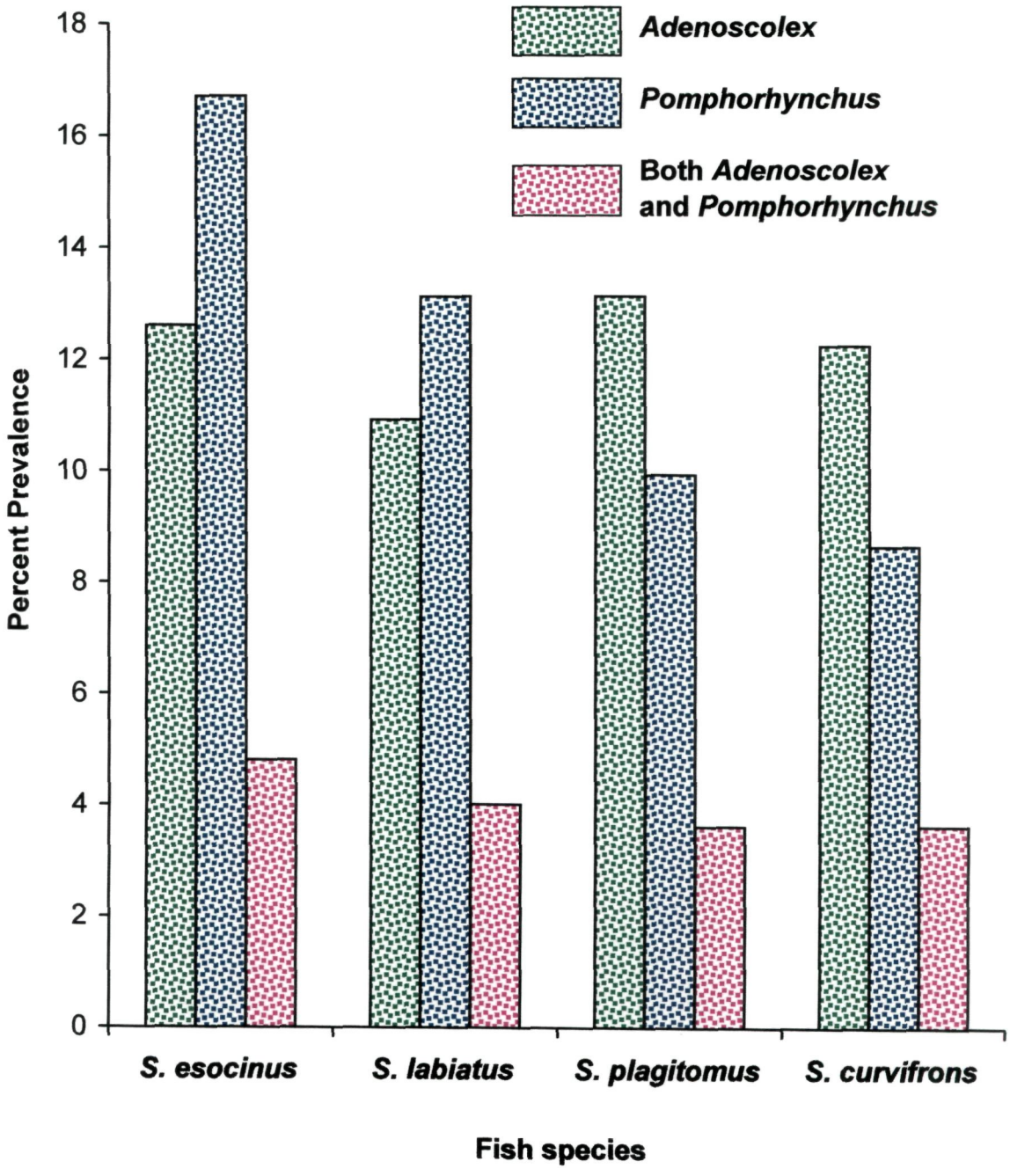


Fig. 9. Percent prevalence of *A. oreini* and *P. kashmirensis* in different species of *Schizothorax*.

Table 5. Seasonal variations in the prevalence of *A. oreini* and *P. kashmirensis* in *Schizothorax* species collected from Kashmir Valley.

Seasons	Total no. of fish examined	Total no. of fish infected	No. of fish having only *A. oreini* infection	No. of fish having only *P. kashmirensis* infection	No. of fish having Both *A. oreini* and *P. kashmirensis* infection
Summer	232	30 (12.93%)	25 (83.3%)	5 (16.7%)	–
Autumn	218	50 (22.90%)	19 (38.0%)	28 (56.0%)	3 (6.0%)
Winter	309	111 (35.92%)	42 (37.8%)	46 (41.4%)	23 (20.7%)
Spring	243	94 (38.68%)	37 (39.4%)	42 (44.7%)	15 (15.95%)

Values in parenthesis show the percent prevalence.

picture. The prevalence of *A. oreini* was found higher during summer whereas, *P. kashmirensis* during autumn. Mixed infections of these parasites were found in all seasons except during summer (Fig. 10). Analysis of the data on monthly basis reveal that the infection of *A. oreini* was maximum during March and lowest during November, but *P. kashmirensis* infection was higher during November, while there was no infection during August (Table 6, Fig. 11).

The data of present study was also analyzed on the basis of sex and length of the fish host. It was observed that length has more influence on the prevalence of *A. oreini* and *P. kashmirensis* as compared to sex of the host, as slightly more males were found infected than females (Table 7). The prevalence and intensity of infection with these parasites increased with increasing size of the fishes and then decreases. The fishes having 20 to 30 cm body length were found more infected (Fig. 12 A). The maximum intensity of infection with *P. kashmirensis* and *A. oreini* was found in the fishes having 25 to 30 and 35 to 40 cm body length, respectively (Fig. 12 B). The intensity was not observed on the basis of sex of the host, therefore, further studies are required.

The frequency distribution of *A. oreini* and *P. kashmirensis* in fishes was over dispersed. Majority of the fishes harboured 1–15 parasites, however, only few fishes were found heavily infected with these parasites. A total 937 *Adenoscolex* and 505 *Pomphorhynchus* were recovered from 7 infected

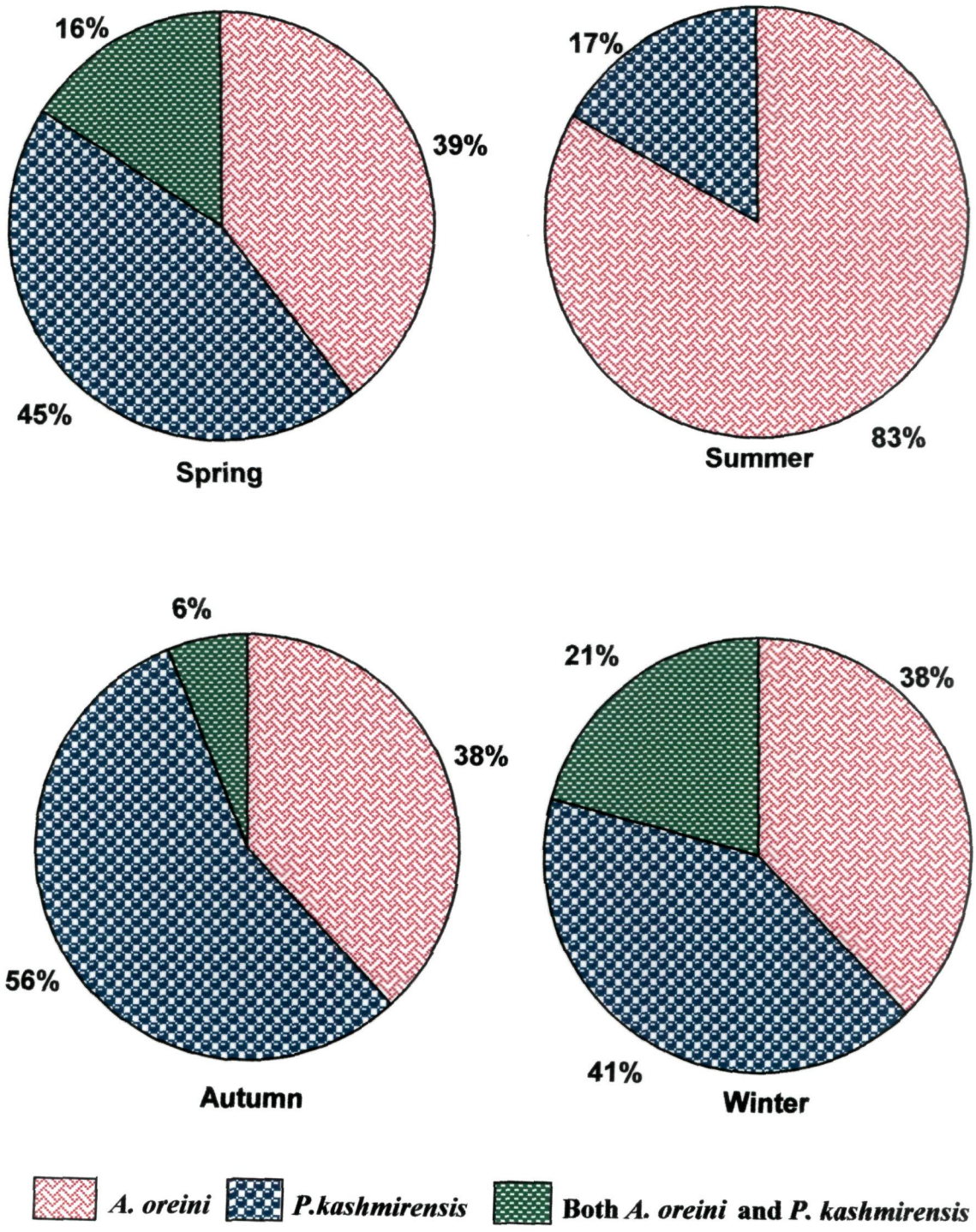


Fig. 10. Seasonal variations in the prevalence of *A. oreini* and *P. kashmirensis* (360° assumed as 100%). Actual values are shown in Table 5.

Table 6. Prevalence of *A. oreini* and *P. kashmirensis* in *Schizothorax* species collected during different months.

Months	Total no. of fish examined	No. of fish having only *A. oreini* infection	Percent prevalence of *A. oreini*	No. of fish having only *P. kashmirensis* infection	Percent prevalence of *P. kashmirensis*	No. of fish having both *A. oreini* and *P. kashmirensis* infection	Percent prevalence of both parasites
June	82	8	9.8	4	5	–	–
July	85	10	11.8	1	1.2	–	–
August	65	7	10.7	–	–	–	–
September	82	8	9.8	3	3.6	–	–
October	49	4	8.2	7	14.3	–	–
November	87	7	8	18	20.7	3	3.4
December	119	14	11.7	15	12.6	8	6.7
January	88	11	12.5	15	17	9	10.2
February	102	17	16.6	16	15.6	6	5.9
March	59	12	20.3	11	18.6	4	6.8
April	104	16	15.4	20	19.2	6	5.8
May	80	9	11.3	11	13.7	5	6.2
Total	1002	123	12.27	121	12.10	41	4.1

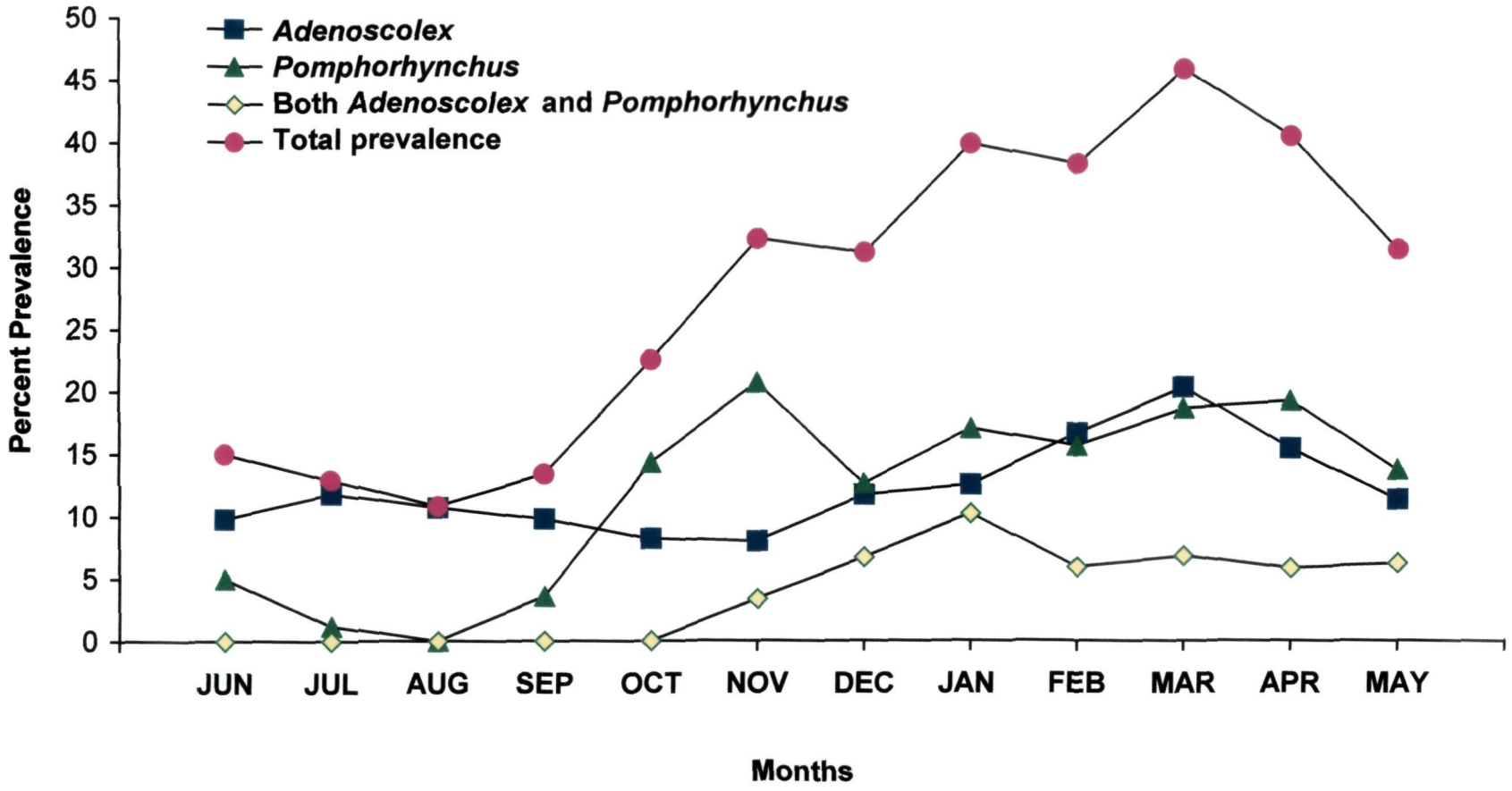


Fig. 11. Percent prevalence of *A. oreini* and *P. kashmirensis* in *Schizothorax* species collected during different months.

Table 7. Prevalence of *A. oreini* and *P. kashmirensis* in different species of *Schizothorax* with respect to sex.

Fish species	No. of fish examined	No. of fish infected		No. of male fish infected			No. of female fish infected		
		Male	Female	A	P	A+P	A	P	A+P
S.curvifrons	320	32	45	15	12	5	24	16	5
S.esocinus	294	38	64	16	18	4	21	31	12
S.labiatus	175	22	27	10	10	2	9	13	5
S.plagitomus	213	24	33	11	10	3	17	11	5
Total	1002	116 (11.6%)	169 (16.9%)	52 (44.8%)	50 (43.1%)	14 (12.1%)	71 (42.01%)	71 (42.01%)	27 (15.98%)

Values in parenthesis represent the percent infection.

A = *Adenoscolex*
P = *Pomphorhynchus*
A+P = Both *Adenoscolex* and *Pomphorhynchus*

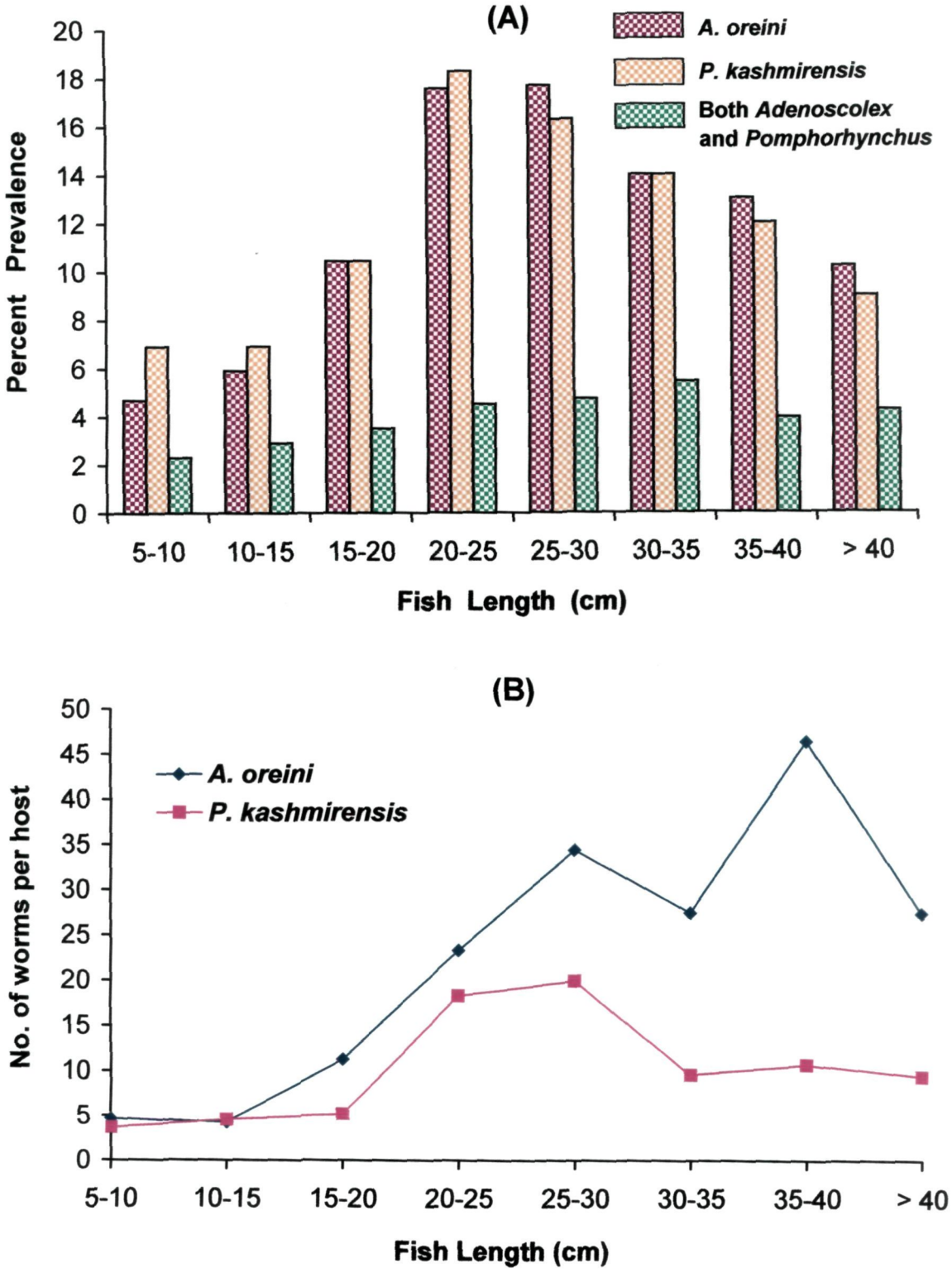


Fig.12. Percent prevalence (A) and intensity of infection (B) of *A. oreini* and *P. kashmirensis* in relation to fish length.

fishes (Figs. 13, 14) which indicate that the fishes were more heavily infected with *A. oreini* as compared to *P. kashmirensis*.

[II] Histopathological Studies:

The foregoing results of epidemiology of *A. oreini* and *P. kashmirensis* have provided an opportunity to monitor the pathological changes in the host's intestine due to these parasites. The results obtained are significant and provide basic information about host–parasite relationship and the host response particularly at the level of cell mediated immunity.

It was observed that *A. oreini* does not make any intimate contact with the intestine of the definitive host but remains in the lumen of the intestine in close vicinity of the villi (Plate 4, Fig. D). In the scolex region of this parasite, certain specialized gland cells were noticed which extend from the scolex in the form of three columns to the three–quarter parts of the body (Plate 1, Fig. D). These cells were round in shape and had well defined cytoplasmic border. Further on comparing the structure of villi of control and infected animals, the first apparent changes were noticed in the absorptive surface. Villi in close vicinity of the scolex become short, compressed and lose their enfoldings leading to decreased surface area for absorption (Plate 4, Figs. A–D). The other important observation was infiltration of the cells which were differentiated as lymphocytes, fibroblasts, neutrophills and plasma cells (Plate 4, Figs. E–H).

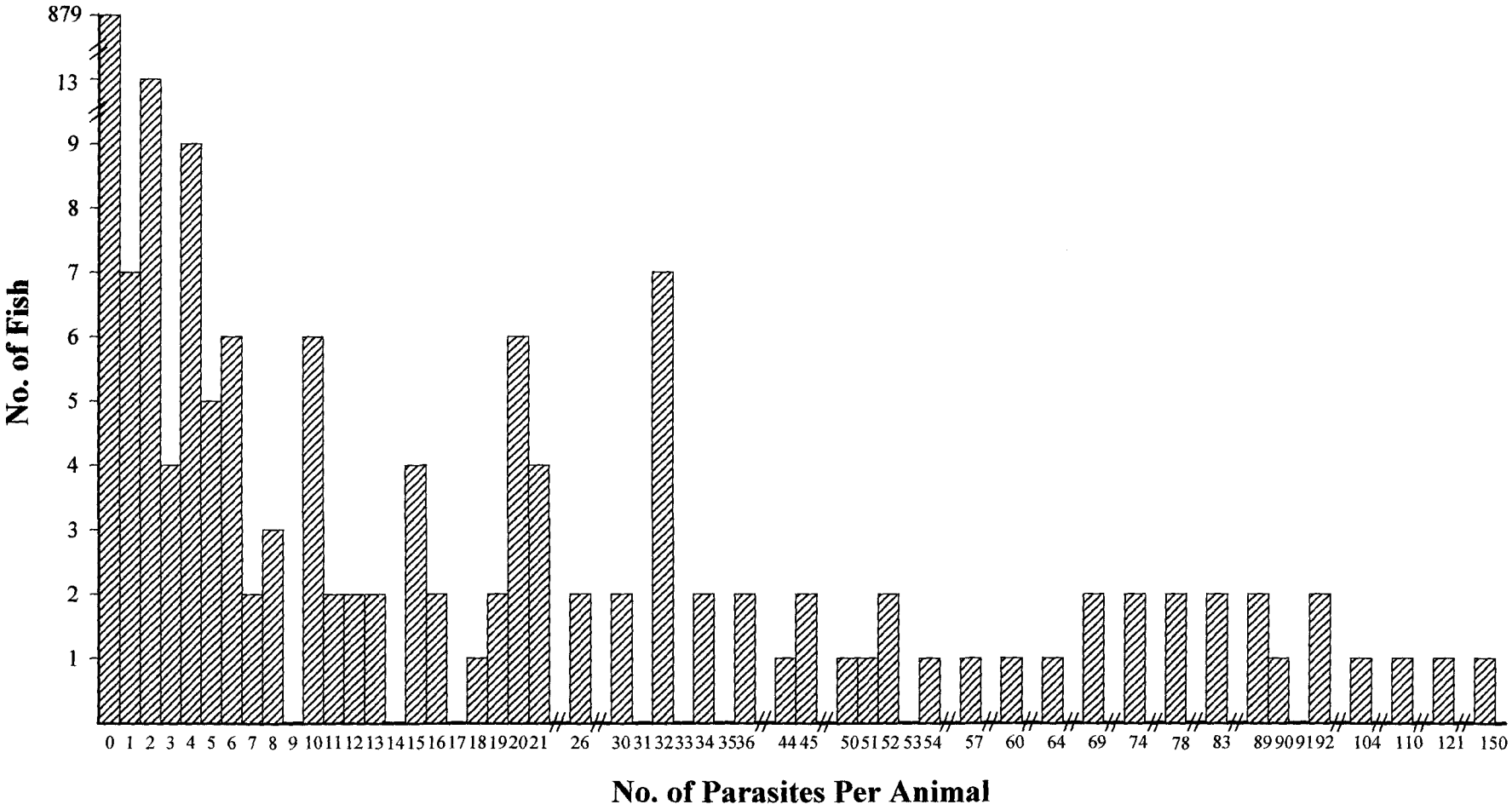


Fig. 13. Frequency distribution of *A. oerini* in *Schizothorax* species.

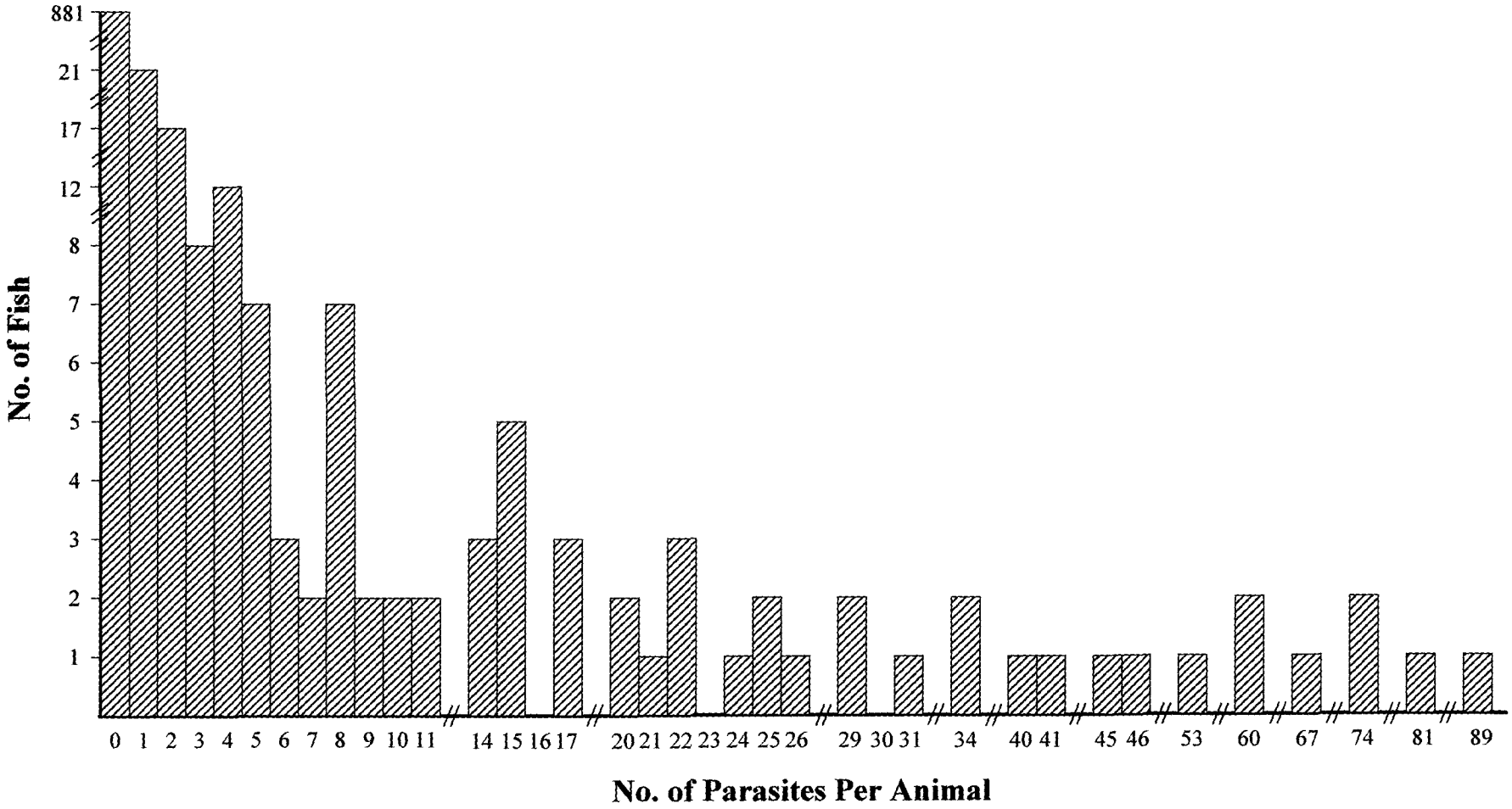


Fig. 14. Frequency distribution of *P. kashmirensis* in *Schizothorax* species.

Plate 4. Sections of non-infected and infected fish intestine with *A. oreini*, showing histopathological changes, stained with eosin and haematoxylin.

Figs.

A. Intestine of non-infected fish showing the size of villi (arrows). [× 100]

B, C & D. Sections of infected intestine, showing changes in the size and structure of villi (arrows), in close vicinity of parasite (P). [× 100]

E & F. Sections of non-infected and infected fish intestine respectively, showing increased cellular infiltration (arrows) at the site of attachment of parasite (P). [×450]

G & H. The infiltrated cells were identified as goblet cells (G), lymphocytes (arrow heads), plasma cell (Pc), fibroblast (F) and neutrophils (N). [× 1000]

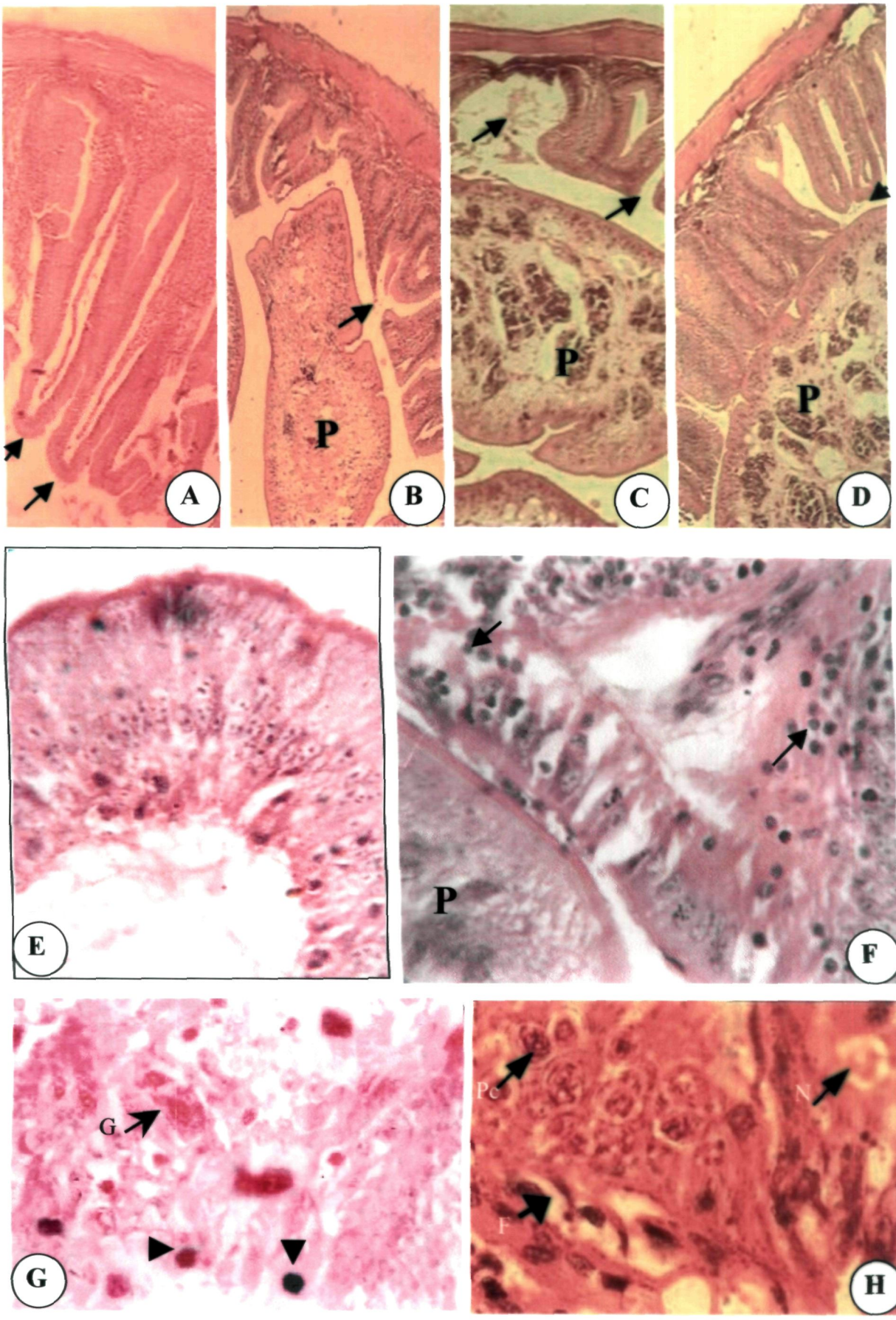


Plate 4

In contrast to *A. oreini*, *P. kashmirensis* make intimate contact and are firmly attached with host intestine by means of long hooked proboscis and bulbous neck. The parasites penetrate their proboscis and bulbous neck deep into the host tissue and thereby damage the villi, epithelial lining, lamina propria and other layers at the site of attachment (Plate 5, Figs. B, C). A large number of fibrous capsules were formed around the proboscis and bulbous neck. Comparison of the villi between non–infected and infected animals reveals that the damage to the villi of infected animals was so severe that it causes total loss of absorptive surface area (Plate 5, Figs. A, B). Extensive proliferation of intestine was observed in heavy infection, which causes the damage to the adjoining tissues of liver and pancreas. Infiltration of cells was noticed at the site of attachment, which gave a granuloma like appearance (Plate 5, Figs. E, F). These infiltrated cells were identified as lymphocytes, fibroblasts and plasma cells (Plate 5, Figs. G, H).

[III] Pathophysiological Studies:

In order to find out the pathophysiological effects, various serum enzymes like transaminases (GOT and GPT), acid and alkaline phosphatases as well as total serum proteins, lipids and their fractions were estimated in the infected as well as non-infected fishes during different seasons. The level of proteins and glycogen was also analyzed in the muscle and liver of non-infected and infected fishes.

Plate 5. Sections of non-infected and infected fish intestine with *P. kashmirensis*, showing histopathological changes, stained with eosin and haematoxylin.

Figs.

A. Intestine of non-infected fish showing the size of villi (arrows). [× 100]

B & C. Sections of infected intestine, showing destruction of villi and lamina propria at the parasite (P) attachment site (arrows). [× 100]

D & E, F. Sections of non-infected and infected fish intestine respectively, showing increased cellular infiltration (arrows) in infected intestine. [×450]

G & H. The infiltrated cells were identified as lymphocytes (arrow), fibroblasts (arrow heads) and plasma cell (Pc). [× 1000]

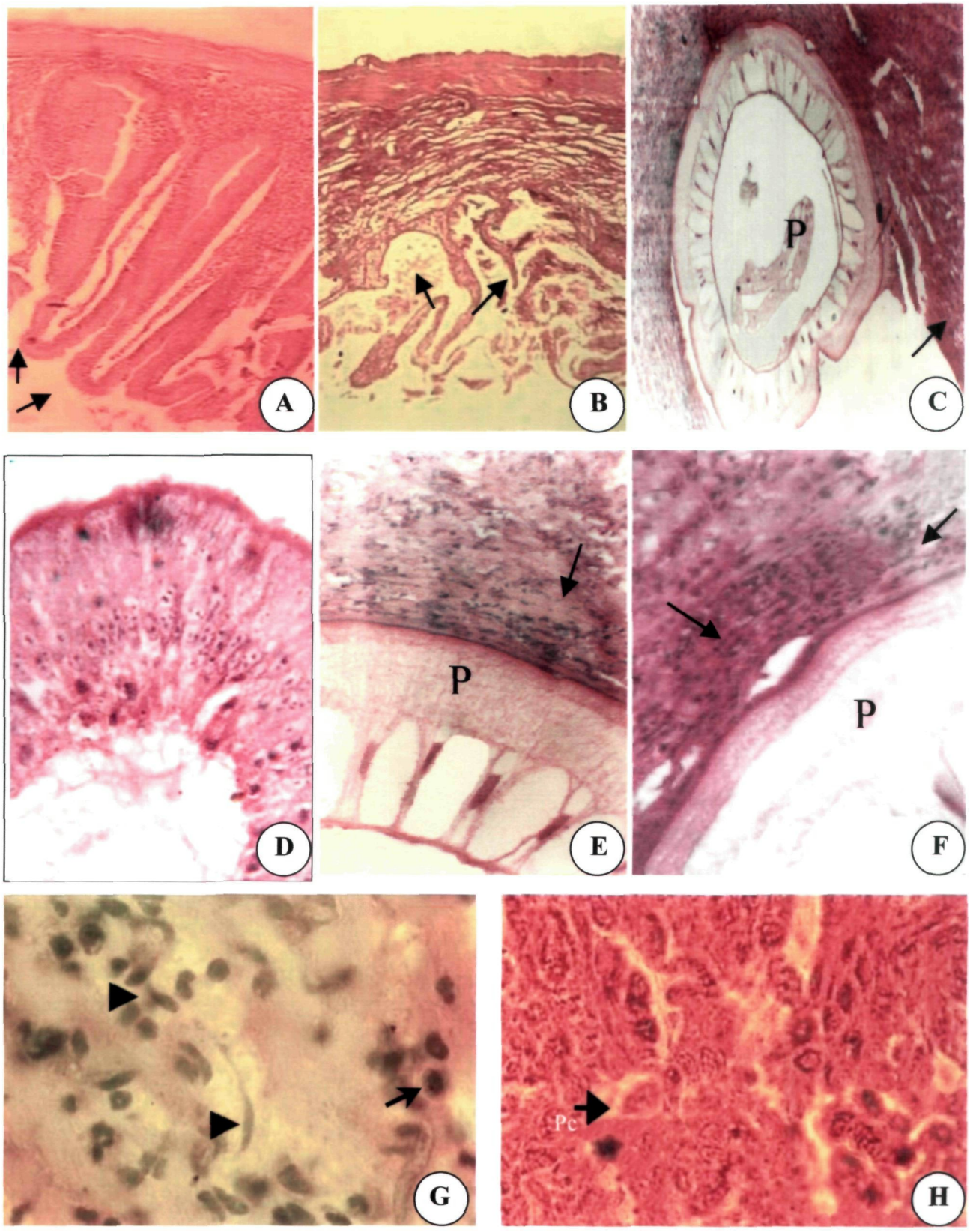


Plate 5

[i] Transaminases: Analysis of serum glutamate oxaloacetate transaminase (sGOT) and serum glutamate pyruvate transaminase (sGPT) revealed that the level of sGOT was found lower than sGPT in both non–infected and infected fishes with *P. kashmirensis* (Table 8) whereas, irregular fluctuation was noticed in *A. oreini* infected fish sera (Table 9). The activity of these enzymes in both non–infected and infected fish sera fluctuates during different seasons and maximum activity was observed during summer (Tables 8, 9).

In order to find out the effect of parasitism on the level of these enzymes, percent change were calculated with their respective controls during different seasons and the results are presented in Fig. 15. It is evident from the Fig. 15A, that the level of sGOT increases while sGPT decreases in *A. oreini* infected fish sera as compared to control during different seasons, and the percent change of sGOT was higher than sGPT. However, in *P. kashmirensis* infected fish sera, the level of both sGOT and sGPT was found higher as compared to control (Fig. 15B). Further, the percent change of sGOT was found exceptionally high during summer and sGPT during summer and spring in *P. kashmirensis* infected fish sera than control (Fig. 15B).

Statistical analysis of the data during different seasons revealed significant fluctuations in the enzyme activity in infected fish sera as compared to control. The activity of sGOT was significantly higher during all seasons except summer whereas, sGPT activity was significantly lower during summer and spring in *A. oreini* infected fish sera (Table 10). In *P. kashmirensis*

Table 8. Biochemical analysis of sera from non-infected and infected *Schizothorax* species with *P. kashmirensis*.

Biochemical Components	Summer	Autumn	Winter	Spring
^ GOT	253.50 ± 9.2 {2} (81.50 ±1.71) {2}	85.00 ± 1.1 {3} (66.20 ± 3.77) {5}	93.00 ± 3.92 {4} (57.00 ± 3.69) {6}	109.20 ± 10.06 {5} (61.00 ± 4.08) {4}
^ GPT	374.00 ± 8.49 {2} (113.67±2.52) {3}	112.75 ± 8.85 {4} (82.50 ± 5.07) {4}	94.57 ± 2.66 {6} (65.50 ± 3.39) {6}	272.00 ± 8.52 {4} (99.75 ± 4.57) {4}
^ Ac Pase	1.38 ± 0.17 {2} (1.60 ± 0.35) {3}	1.36 ± 0.21 {4} (1.52 ± 0.26) {5}	1.63 ± 0.21 {5} (1.94 ± 0.11) {5}	1.54 ± 0.21 {7} (1.86 ± 0.19) {5}
^ Al Pase	65.60 ± 4.11 {2} (74.67± 3.06) {3}	56.20 ± 10.43 {4} (68.40 ± 4.39) {5}	45.33 ± 6.09 {5} (66.33 ± 4.32) {6}	43.75 ± 7.32 {4} (88.75 ± 2.63) {4}
*Total Serum Proteins	5.5 ± 0.37 {5} (7.6 ± 0.28) {11}	3.7 ± 0.08 {12} (5.4 ± 0.22) {4}	4.6 ± 0.17 {7} (6.2 ± 0.19) {5}	5.1 ± 0.21 {8} (7.1 ± 0.28) {12}
* Serum Albumin	2.4 ± 0.24 {6} (4.9 ± 0.15) {10}	1.3 ± 0.06 {5} (3.5 ± 0.11) {5}	1.7 ± 0.26 {6} (4.5 ± 0.17) {3}	1.8 ± 0.06 {4} (4.3 ± 0.15) {3}
* Serum Globulin	3.1 ± 0.25 {3} (2.7 ± 0.17) {5}	2.4 ± 0.29 {5} (1.9 ± 0.10) {4}	2.9 ± 0.37 {6} (1.7 ± 0.19) {4}	3.3 ± 0.30 {7} (2.8 ± 0.27) {6}
$ Serum Total Lipids	667.67 ± 18.34 {3} (538.33±17.62){3}	776.20 ± 17.80 {5} (619.67 ± 9.99){9}	843.00 ± 16.52 {9} (697.00±16.08){5}	690.00 ± 5.93 {6} (566.4±12.67){10}
$ Serum Cholesterol	183.00 ± 4.97 {4} (132.80±11.82){5}	215.00 ± 12.70 {6} (157.29 ± 7.83){7}	283.00 ± 8.08 {12} (212.89±8.67) {9}	248.50±13.54 {10} (180.91±6.09){11}
$ Serum Triglycerides	207.00 ± 8.49 {2} (166.25±2.06) {4}	246.11 ± 14.58 {9} (180.89 ± 7.83){9}	350.57 ± 19.42 {7} (262.00±13.24){8}	276.42±14.48 {12} (217.09±14.5){11}
$ HDL	60.00 ± 1.64 {2} (51.00 ± 4.24) {2}	66.00 ± 2.83 {2} (61.00 ± 3.03) {3}	90.00 ± 4.47 {4} (81.25 ± 4.03) {4}	76.25 ± 1.71 {3} (71.50 ± 2.12) {2}
$ LDL	81.60 ± 1.6 {2} (48.55 ± 4.7) {2}	99.80 ± 8.7 {2} (60.11 ± 5.9) {3}	123.31 ± 8.8 {3} (79.24 ± 8.1) {4}	116.97 ± 11.3 {4} (65.99 ± 3.7) {2}

^ All values are expressed as IU/L of sera ± SEM.
* All values are expressed as g/100 ml of sera ± SEM.
$ All values are expressed as mg/100 ml of sera ± SEM.
Values in parenthesis represent control.
Values in curved bracket {} represent the total number of samples analyzed.

Table 9. Biochemical analysis of sera from non-infected and infected *Schizothorax* species with *A. oreini.*

Biochemical Components	Summer	Autumn	Winter	Spring
^ GOT	84.00 ±2.01 {2} (81.50 ±1.71) {3}	78.33 ± 3.79 {3} (66.20 ± 3.77) {5}	63.80 ± 5.07 {5} (57.00 ± 3.69) {6}	76.40 ± 2.30 {5} (61.00 ± 4.08) {4}
^ GPT	85.67 ± 3.51 {3} (113.67 ± 2.52){3}	75.60 ± 2.70 {5} (82.50 ± 5.07) {4}	61.86 ± 2.41 {7} (65.50 ± 3.39) {6}	82.25 ± 2.75 {4} (99.75 ± 4.57) {4}
^ Ac Pase	2.30 ± 0.26 {4} (1.60 ± 0.35) {3}	2.43 ± 0.28 {4} (1.52 ± 0.26) {5}	2.40 ± 0.27 {4} (1.94 ± 0.11) {5}	2.40 ± 0.28 {6} (1.86 ± 0.19) {5}
^ Al Pase	89.75 ± 4.35 {4} (74.67± 3.06) {3}	86.00 ± 3.94 {5} (68.40 ± 4.39) {5}	85.50 ± 4.20 {4} (66.33 ± 4.32) {6}	94 ± 3.16 {5} (88.75 ± 2.63) {4}
*Total Serum Proteins	5.2 ± 0.28 {10} (7.6 ± 0.28) {11}	4.3 ± 0.14 {8} (5.4 ± 0.22) {4}	4.6 ± 0.50 {5} (6.2 ± 0.19) {5}	5.5 ± 0.22 {9} (7.1 ± 0.28) {12}
* Serum Albumin	1.4 ± 0.35 {5} (4.9 ± 0.15) {10}	2.2 ± 0.14 {4} (3.5 ± 0.11) {5}	2.3 ± 0.23 {6} (4.5 ± 0.17) {3}	1.8 ± 0.16 {6} (4.3 ± 0.15) {3}
* Serum Globulin	3.8 ± 0.20 {3} (2.7 ± 0.17) {5}	2.1 ± 0.21 {5} (1.9 ± 0.10) {4}	2.3 ± 0.24 {7} (1.7 ± 0.19) {4}	3.7 ± 0.15 {6} (2.8 ± 0.27) {6}
$ Serum Total Lipids	679.00 ±13.45 {3} (538.33±17.62 {3}	770.43 ± 20.88 {7} (619.67 ± 9.99){9}	864.71 ± 17.93 {7} (697.00±16.08){5}	730.67 ± 16.56 {6} (566.4±12.67){10}
$ Serum Cholesterol	212.17 ± 7.31 {6} (132.80±11.82){5}	250.25 ± 18.52 {8} (157.29 ±7.83) {7}	332.29 ± 19.16 {7} (212.89±8.67) {9}	289.10 ± 13.0{10} (180.91±6.09){11}
$ Serum Triglycerides	224.00 ± 14.14 {2} (166.25 ± 2.06){4}	291.38 ± 19.65 {8} (180.89 ±7.83) {9}	431.20±20.19 {10} (262.0±13.24) {8}	362.00 ± 19.7{12} (217.09±14.5){11}
$ HDL	28.5 ± 1.71 {2} (51.00 ± 4.24) {2}	34.00 ± 2.01 {3} (61.00 ± 3.03) {3}	56.00 ± 2.02 {3} (81.25 ± 4.03) {4}	44.3 ± 2.52 {3} (71.50 ± 2.12) {2}
$ LDL	138.90 ± 1.4 {2} (48.55 ± 4.7) {2}	157.97 ± 7.0 {3} (60.11 ± 5.9) {3}	190.05 ± 1.5 {3} (79.24 ± 8.1) {4}	172.37 ± 12.5 {3} (65.99 ± 3.7) {2}

^ All values are expressed as IU/L of sera ± SEM.
* All values are expressed as g/100 ml of sera ± SEM.
$ All values are expressed as mg/100 ml of sera ± SEM.
Values in parenthesis represent control.
Values in curved bracket {} represent the total number of samples analyzed.

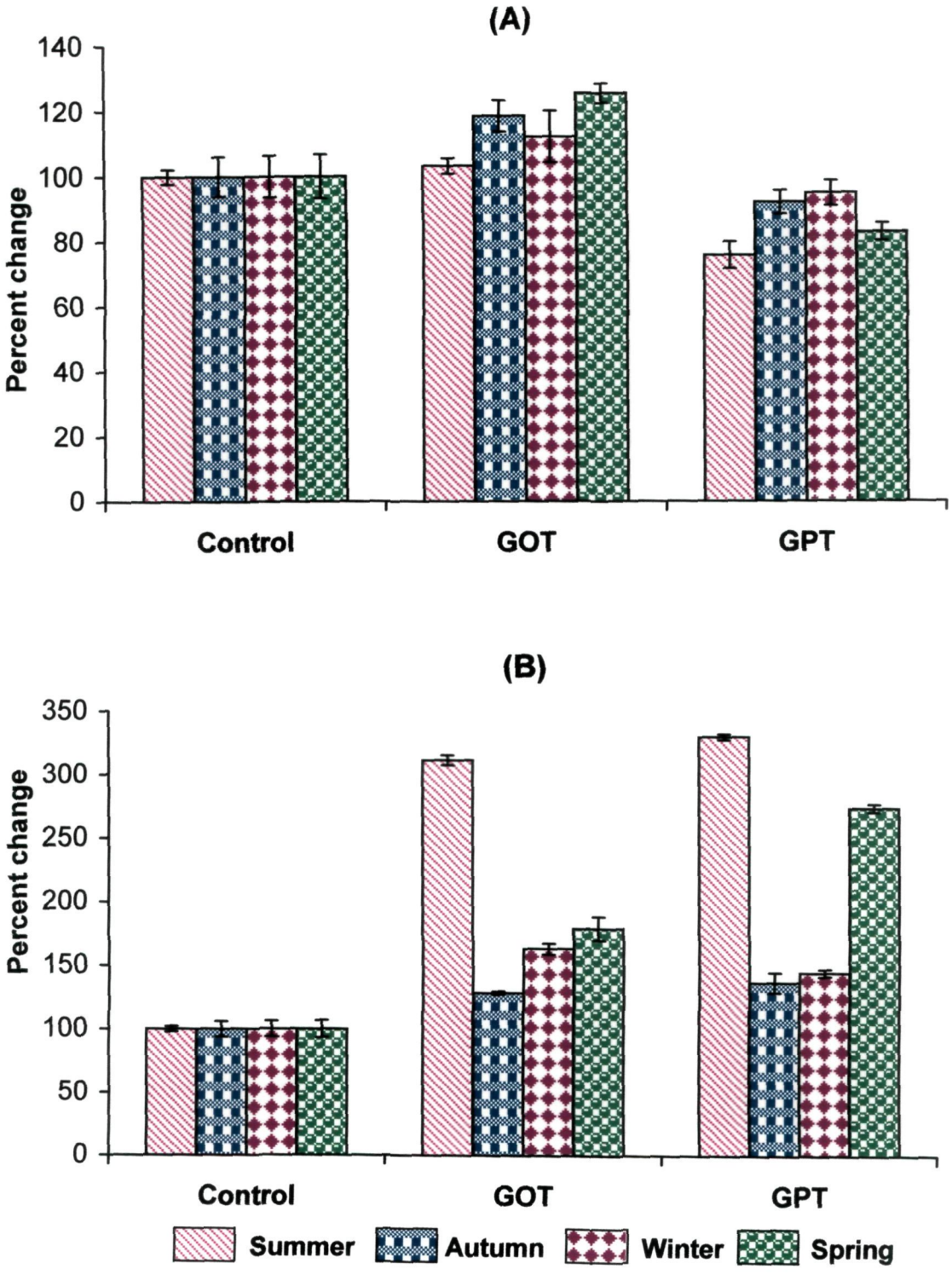


Fig. 15. Percent change of serum glutamate oxaloacatate transaminase (GOT) and glutamate pyruvate transaminase (GPT) in *Schizothorax* species infected with *A. oreini* (A) and *P. kashmirensis* (B) at different seasons

Table 10. Level of statistical significance (*P* value) of various blood components of *Schizothorax* species infected with *A. oreini.*

Seasons	GOT	GPT	AC Pase	Al Pase	Total Serum Protein	Serum Albumin	Serum Globulin	Serum total lipids	Serum cholesterol	Serum Triglycerides	HDL	LDL	PCV	Hb
Summer	< 0.30	< 0.01	< 0.05	< 0.01	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.05	< 0.01	< 0.50	< 0.1
Autumn	< 0.05	< 0.1	< 0.01	< 0.001	< 0.001	< 0.001	< 0.05	< 0.001	< 0.001	< 0.001	< 0.001	< 0.01	< 0.05	< 0.1
Winter	< 0.05	< 0.1	< 0.01	< 0.001	< 0.001	< 0.001	< 0.01	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.05	< 0.1
Spring	< 0.001	< 0.01	< 0.01	< 0.5	< 0.001	< 0.001	< 0.01	< 0.001	< 0.001	< 0.001	< 0.001	< 0.05	< 0.3	< 0.1

P value up to < 0.05 is considered as significant

infected fish sera, both the enzymes were found significantly higher during all seasons except autumn, where the differences between sGOT level of infected and control sera were found insignificant (Table 11). It is evident from the Tables 10 and 11 that the level of significance varies in each case.

[ii] Acid and Alkaline Phosphatases : The mean serum acid phosphatase activity in infected and non– infected animals was found lower as compared to alkaline phosphatase. It was further observed that the mean activity of both acid and alkaline phosphatases was higher in *A. oreini* whereas, lower in *P. kashmirensis* infected fish sera in all seasons as compared to their respective control (Tables 8, 9).

When percent change of serum acid and alkaline phosphatases were calculated with their respective controls during different seasons, it was observed that both acid and alkaline phosphatases increase in *A. oreini* infected fish sera whereas, decreases in the *P. kashmirensis* infected sera (Fig. 16 A, B).

Statistical analysis of the data revealed that the activity of both acid and alkaline phosphatases increase significantly during all seasons in *A. oreini* infected sera except during spring where, the differences in alkaline phosphatase was found insignificant (Table 10). However, significant decrease in these serum enzymes were found in *P. kashmirensis* infected fishes during all seasons except summer and autumn where the differences in acid

Table 11. Level of statistical significance (*P* value) of various blood components of *Schizothorax* species infected with *P. kashmirensis*.

Seasons	GOT	GPT	AC Pase	Al Pase	Total Serum Protein	Serum Albumin	Serum Globulin	Serum total lipids	Serum Cholesterol	Serum Triglyceride	HDL	LDL	PCV	Hb
Summer	< 0.001	< 0.001	< 0.1	< 0.05	< 0.001	< 0.001	< 0.05	< 0.001	< 0.001	< 0.001	< 0.1	< 0.01	< 0.1	< 0.30
Autumn	< 0.1	< 0.001	< 0.1	< 0.05	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.1	< 0.01	< 0.05	< 0.30
Winter	< 0.001	< 0.01	< 0.05	< 0.001	< 0.001	< 0.001	< 0.05	< 0.001	< 0.001	< 0.001	< 0.1	< 0.05	< 0.01	< 0.50
Spring	< 0.001	< 0.01	< 0.01	< 0.001	< 0.001	< 0.001	< 0.05	< 0.001	< 0.001	< 0.001	< 0.1	< 0.01	< 0.1	< 0.50

P value up to < 0.05 is considered as significant

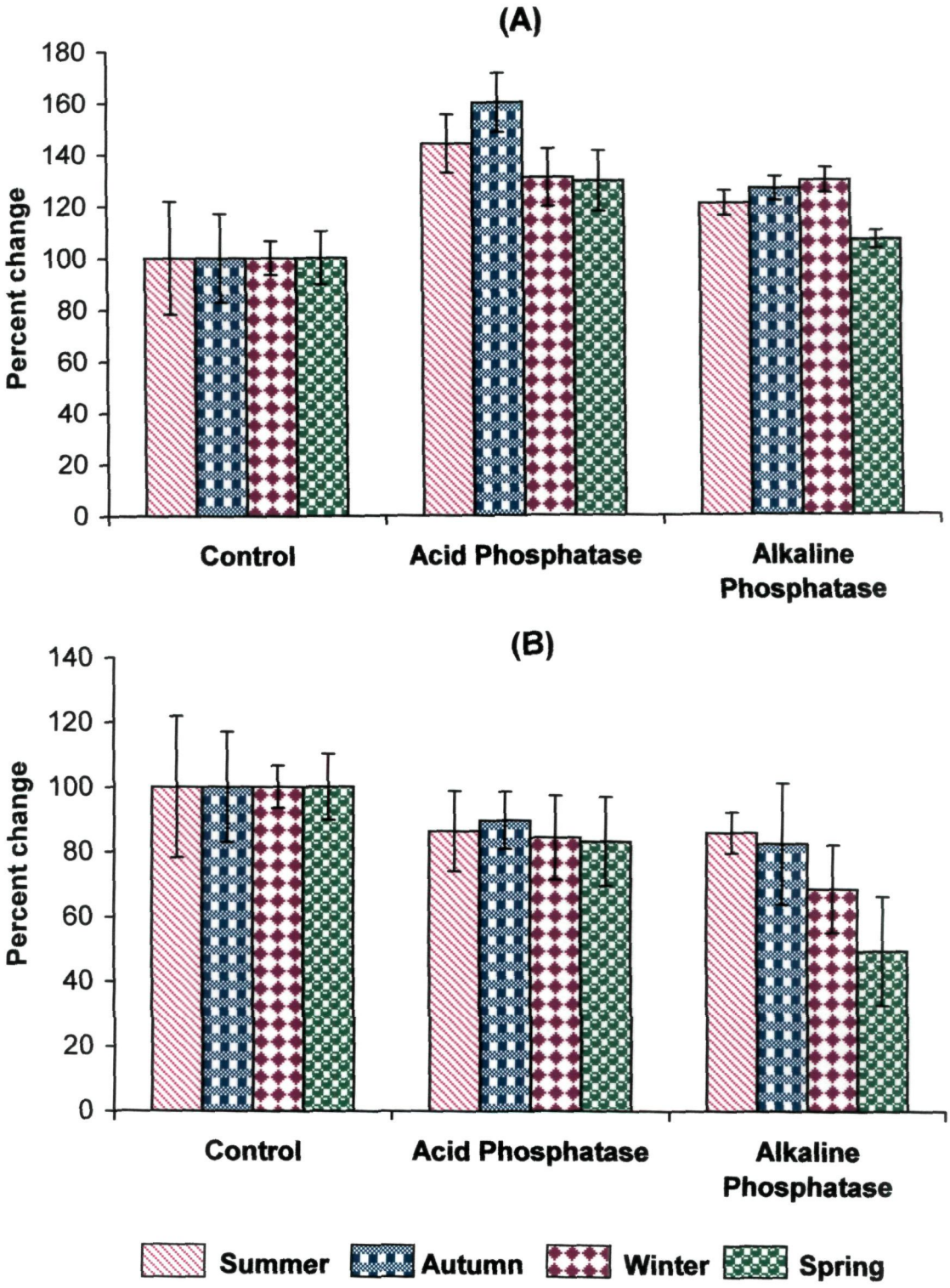


Fig.16. Percent change of serum acid and alkaline phosphatases in *Schizothorax* species infected with *A. oreini* (A) and *P. kashmirensis* (B) at different seasons.

phosphatase were insignificant (Table 11). Maximum decrease in alkaline phosphatase was observed during spring in *P. kashmirensis* infected fish sera (Fig. 16 B).

[iii] Analysis of Serum Proteins: The total serum proteins and various fractions from fishes, non–infected and infected with *A. oreini* and *P. kashmirensis* were also analyzed during different seasons and the results are presented in Tables 8, 9. Further, percent change of total serum proteins, albumin and globulin were also calculated with respect to control which are presented in Figs. 17 A, B. It is evident from the results that total serum proteins and albumin significantly decrease while, globulin significantly increases with respect to control during all seasons. Maximum percent decrease in the level of albumin and increase in the level of globulin was found during summer and autumn due to *A. oreini* and *P. kashmirensis* infection, respectively (Fig. 17 A, B). The level of significance (p–value) is presented in Tables 10, 11.

[iv] Analysis of Serum Lipids: The mean total lipids in infected fish sera was found significantly (Tables 10, 11) higher than controls during all seasons (Tables 8, 9). Maximum percent increase with respect to control was observed during spring and autumn in *A. oreini* and *P. kashmirensis* infections, respectively (Figs. 18, 19). The total serum lipids were further fractionated into cholesterol, triglycerides, HDL and LDL among which, all the components except HDL increase significantly with respect to their controls in all seasons

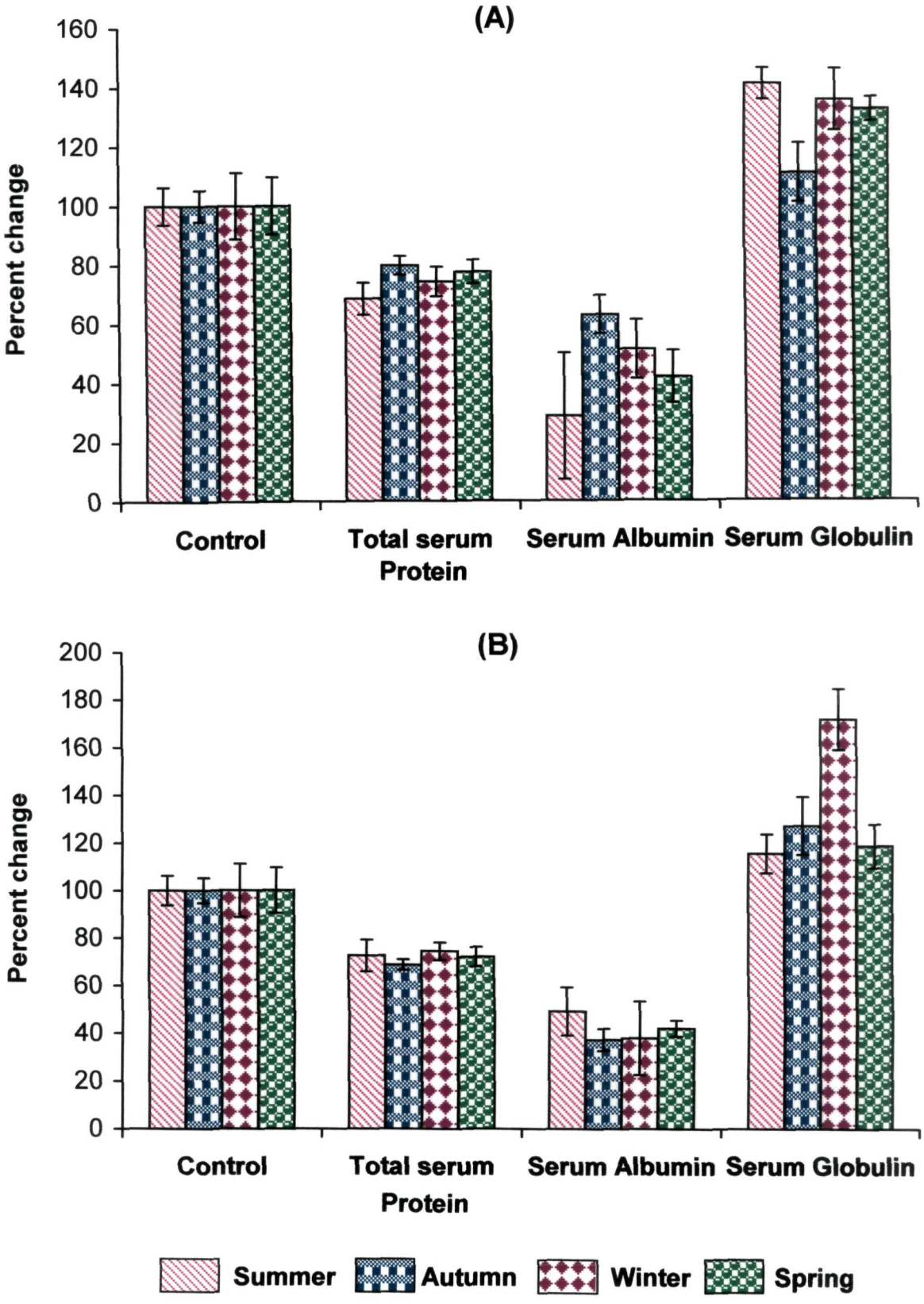


Fig.17. Percent change of total serum proteins and their fractions in *Schizothorax* species infected with *A. oreini* (A) and *P. kashmirensis* (B) at different seasons.

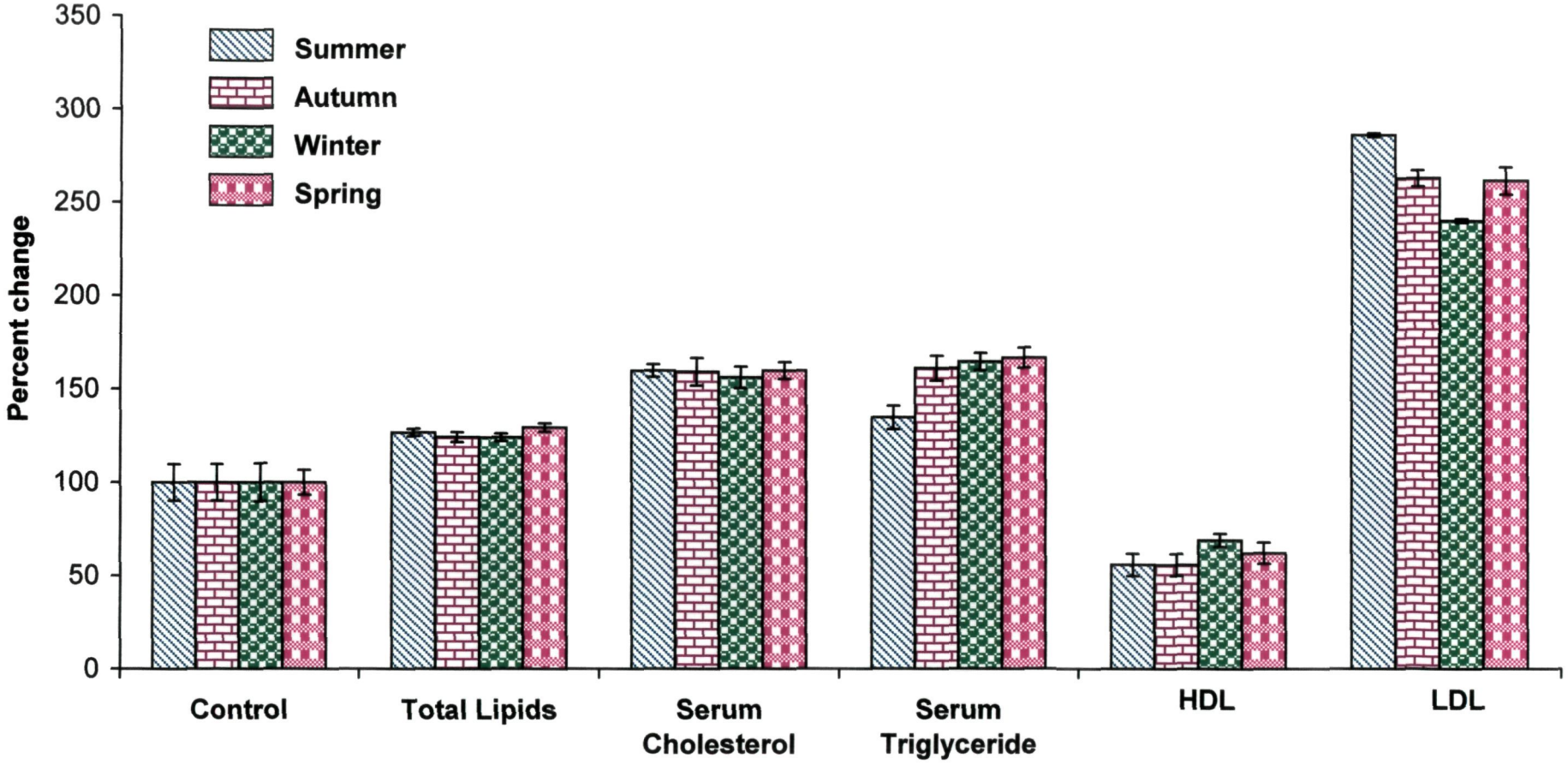


Fig.18. Percent change of serum lipids and their fractions in *Schizothorax species infected with A. oreini at* different seasons.

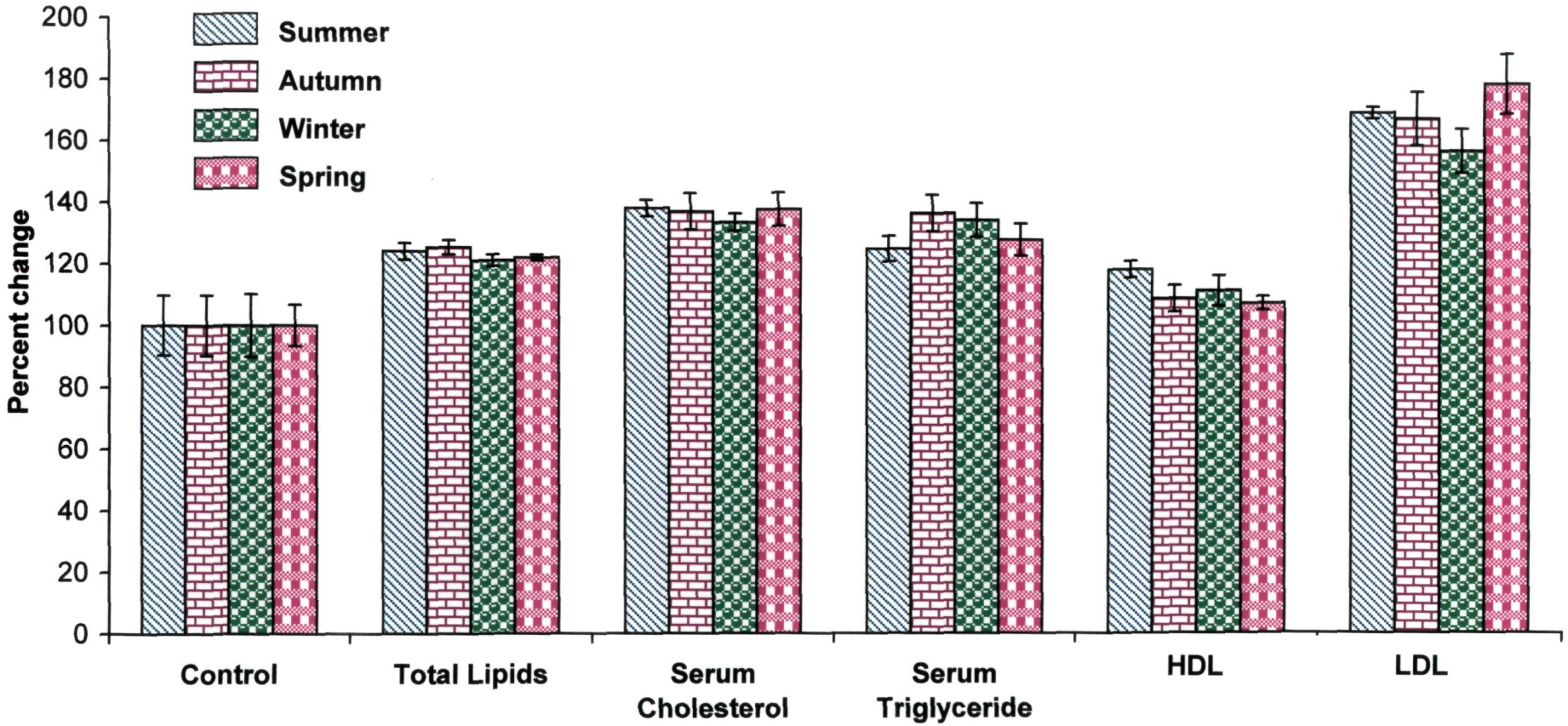


Fig.19. Percent change of serum lipids and their fractions in *Schizothorax* species infected with *P. kashmirensis at* different seasons.

due to infection of both parasites (Tables 8, 9; Figs. 18, 19). The HDL was found significantly low in *A. oreini* (Table 10) and insignificantly higher in *P. kashmirensis* infected fish sera (Table 11). Comparison of the percent change of serum lipids and various fractions during different seasons revealed that all the components were more or less same except triglycerides in *A. oreini* infected sera which was significantly ($p < 0.05$) less during summer as compared to other seasons.

[v] Analysis of Proteins and Glycogen from infected fish muscle and liver: Total proteins and glycogen in the muscle and liver of infected and non-infected fishes were analyzed and the results are presented in Table 12. It is evident from the results that both proteins and glycogen of infected muscle and liver decreases due to infection with both the parasites, and the effect was more pronounced in muscle than liver. Maximum decrease was noticed in the level of glycogen followed by proteins in *A. oreini* infected fish muscle, whereas, in *P. kashmirensis* infection, maximum decrease was observed in the level of muscle proteins followed by liver glycogen (Fig. 20). The differences between these components of infected and non-infected fishes were found significant except glycogen of liver in *A. oreini* infected fishes. The level of significance varies with the biochemical components and parasitic infection (Table 12).

Table 12. Analysis of protein and glycogen from infected muscle and liver of *Schizothorax* species.

Components	Non-infected fish	*A. oreini* infected fish	*P. kashmirensis* infected fish
Muscle Protein	29.4 ± 1.8	20.6 ± 1.1 ($P < 0.001$)	16.8 ± 1.1 ($P < 0.001$)
Muscle Glycogen	12.4 ± 1.6	4.0 ± 0.7 ($P < 0.001$)	10.6 ± 1.1 ($P < 0.05$)
Liver Protein	17.6 ± 1.3	15.8 ± 0.7 ($P < 0.01$)	13.1 ± 1.9 ($P < 0.001$)
Liver Glycogen	7.7 ± 1.2	7.2 ± 1.5 ($P < 0.5$)	5.5 ± 0.6 ($P < 0.01$)

All values are expressed as mg/g wet weight of tissue ± SEM of seven different populations. P values upto 0.05 is considered as significant.

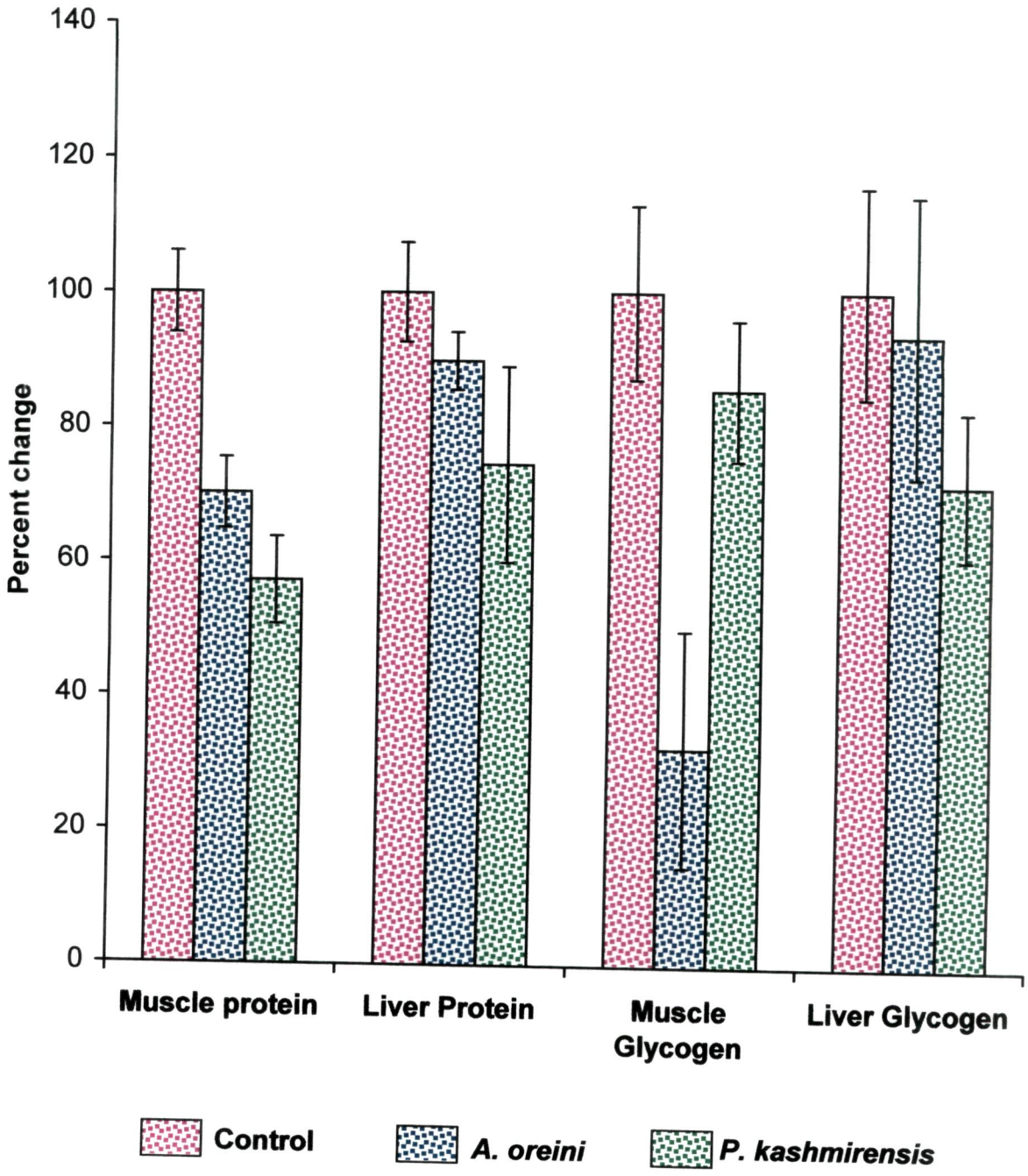


Fig.20. Percent change of protein and glycogen from infected muscle and liver of *Schizothorax* species.

[IV] Haematological Studies:

[i] Total RBC Count: The RBC were counted from both non–infected and infected fishes with *A. oreini* and *P. kashmirensis* and the percent change with respect to control is presented in Fig. 21. It is evident from the results that the level of RBC decreases in both *A. oreini* and *P. kashmirensis* infected fishes and the differences were found statistically insignificant (Table 13).

[ii] Total and Differential Leucocyte Count: The total leucocyte counts were found higher in infected than non–infected fishes (Fig. 21) and the differences were statistically insignificant (Table 13). About 5 % increase was observed in infected fishes with both the parasites as compared to control.

The differential leucocyte counts were also carried out in non–infected and infected fishes with *A. oreini* and *P. kashmirensis*. It was observed that the levels of lymphocytes and neutrophils increase by 17.4% and 23.3%, respectively, while, other cells like thrombocytes, basophils, monocytes and blast cells decrease by about 1–25 % due to *A. oreini* infection. In *P. kashmirensis* infected fishes, lymphocytes, neutrophils and monocytes increased by 20.4%, 34% and 4.5%, respectively, while other cells decreased by 2–20% (Fig. 21 A, B). Such changes in the blood cells were found statistically insignificant (Table 13).

Analysis of the data revealed that seasons have no effect on the total and differential count of blood cells therefore, these data were pooled and presented in the Fig. 21 A, B.

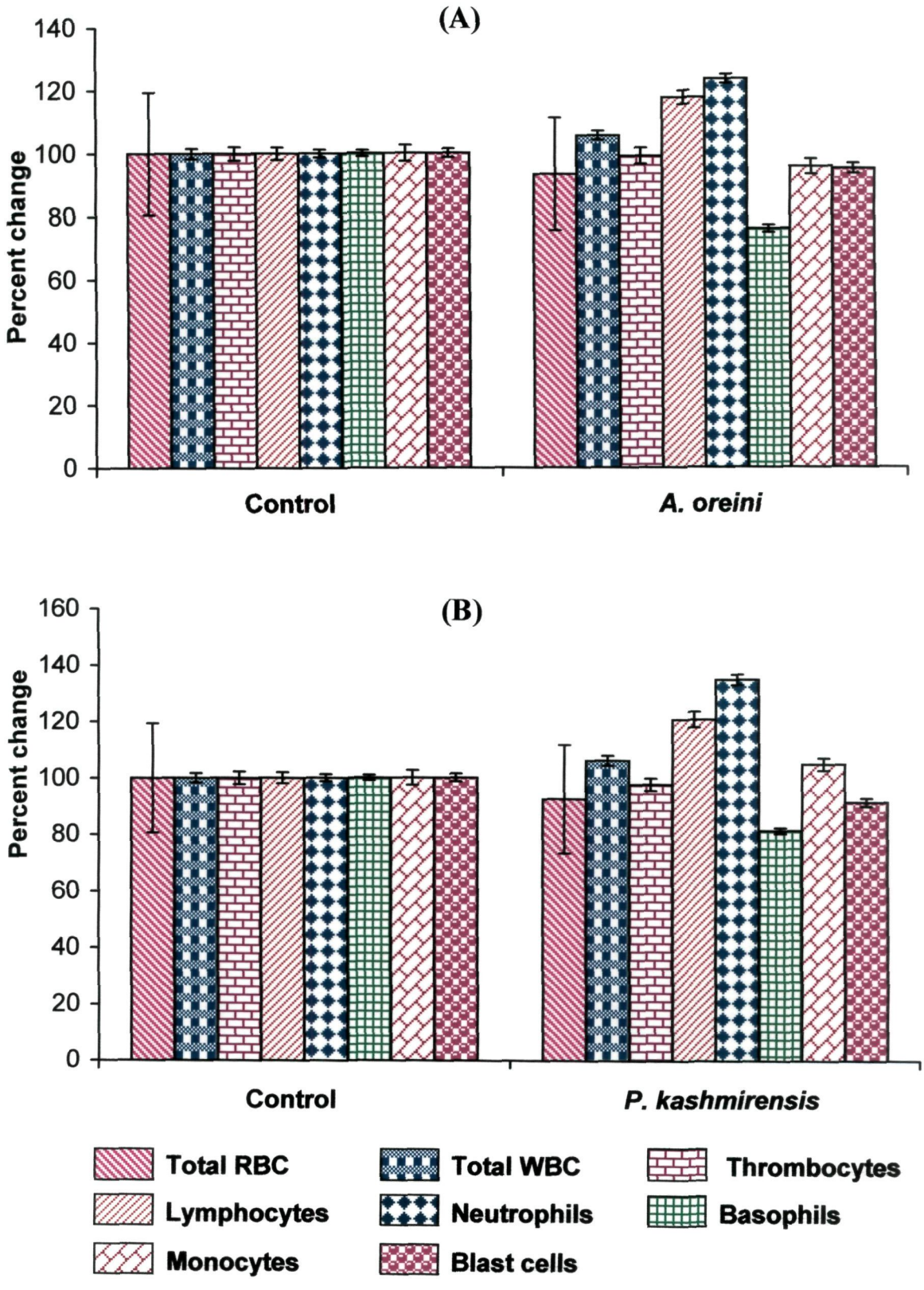


Fig. 21. Percent change of total RBC, WBC and differential leucocyte counts in *Schizothorax* species infected with *A.oreini* (A) and *P. kashmirensis* (B).

Table 13. Level of statistical significance (*P* value) of blood cells of infected *Schizothorax* species.

Blood Cells	*P* value of *A. oreini* infection	*P* value of *P. kashmirensis* infection
RBC	< 0.70	< 0.70
WBC	< 0.70	< 0.70
Thrombocytes	< 0.90	< 0.90
Lymphocytes	< 0.70	< 0.50
Neutrophills	< 0.90	< 0.90
Basophills	< 0.90	< 0.90
Monocytes	< 0.50	< 0.50
Haemoblasts	< 0.90	< 0.90

P value upto <0.05 is considered as significant

[iii] Packed Cell Volume: The packed cell volume (PCV) in infected fishes with both parasites was found slightly higher than non–infected fishes in all seasons (Fig. 22). The PCV was found comparatively higher in *P. kashmirensis* than *A. oreini* infected fishes. In both *A. oreini* and *P. kashmirensis* infected fishes, significant increase was observed during autumn and winter (Tables 10, 11).

[iv] Haemoglobin Content: The percent change in haemoglobin concentrations between infected and non–infected fishes is shown in Fig. 22. The haemoglobin concentrations in the infected fishes with both parasites were found less as compared to non–infected fishes. Statistically insignificant differences were observed between the haemoglobin concentrations of infected and control fishes (Tables 10, 11).

[V] Biochemical Composition and Protein Polymorphism of *Adenoscolex* and *Pomphorhynchus*:

The results of biochemical composition of *A. oreini* and *P. kashmirensis* are presented in Table 14 and Fig. 23. Among various biochemical components, the level of lipids was found higher followed by glycogen in *A. oreini* whereas, the level of glycogen was slightly higher than lipids in *P. kashmirensis* (Fig. 23). The ratio of DNA to that of RNA was calculated as 1: 6 in *A. oreini* and 1: 3.5 in *P. kashmirensis* whereas, the ratio of RNA to protein was found about 1: 4 for both the parasites. Comparison of

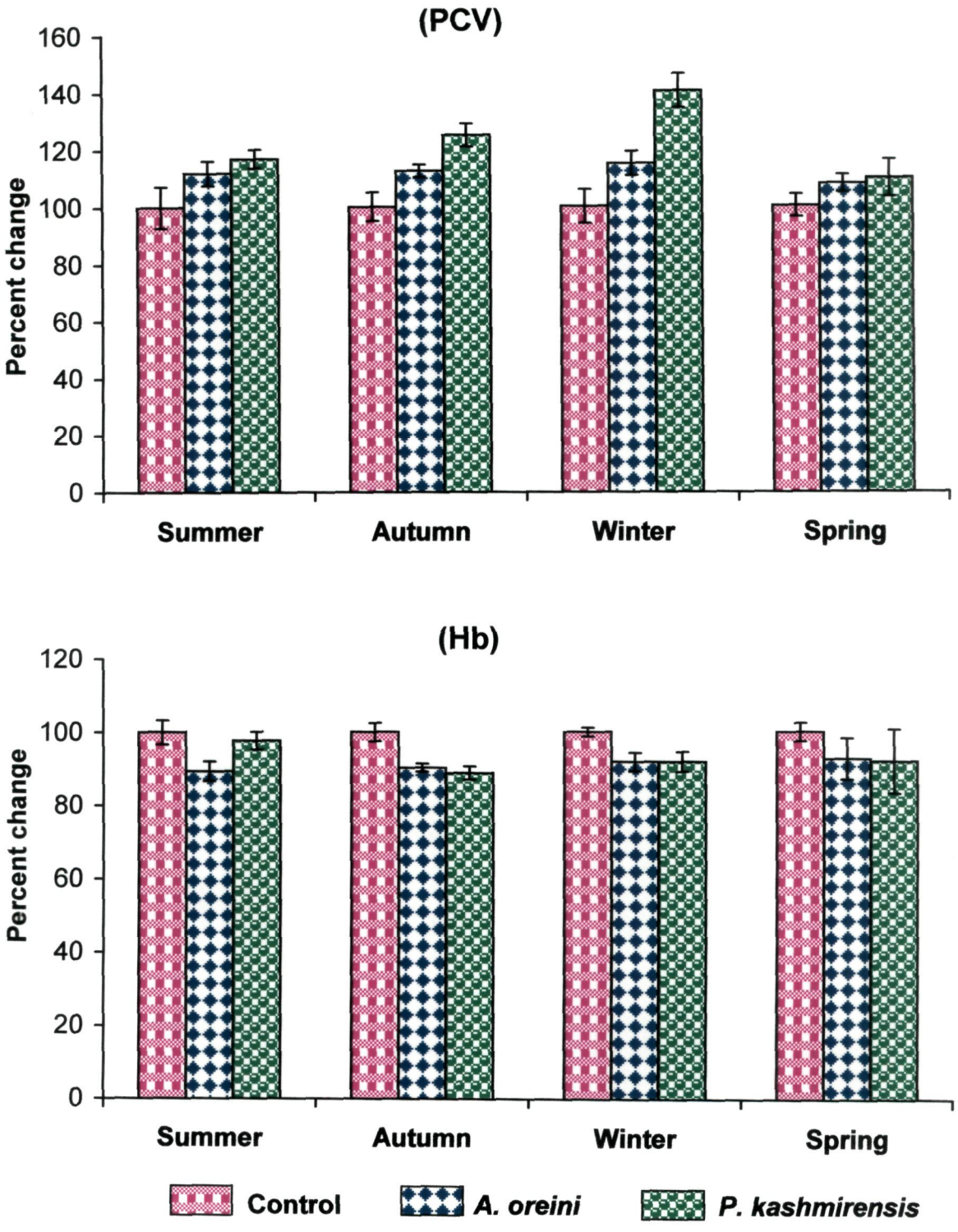


Fig. 22. Percent change of packed cell volume (PCV) and haemoglobin (Hb) in *Schizothorax* species infected with *A. oreini* and *P. kashmirensis* at different seasons.

Table 14. Biochemical composition of *A. oreini* and *P. kashmirensis* isolated from the intestine of *Schizothorax* species.

Biochemical components	*A. oreini*	*P. kashmirensis*	P value
Glycogen	62.34 ± 3.06	97.00 ± 2.95	< 0.001
Proteins	54.11 ± 2.52	79.00 ± 5.13	< 0.001
Lipids	68.74 ± 6.43	94.00 ± 2.52	< 0.001
RNA	12.54 ± 2.53	17.75 ± 0.96	< 0.01
DNA	2.14 ± 0.10	5.02 ± 0.68	< 0.001

All values are expressed as mg/g wet weight of tissue ± SEM of five different populations.

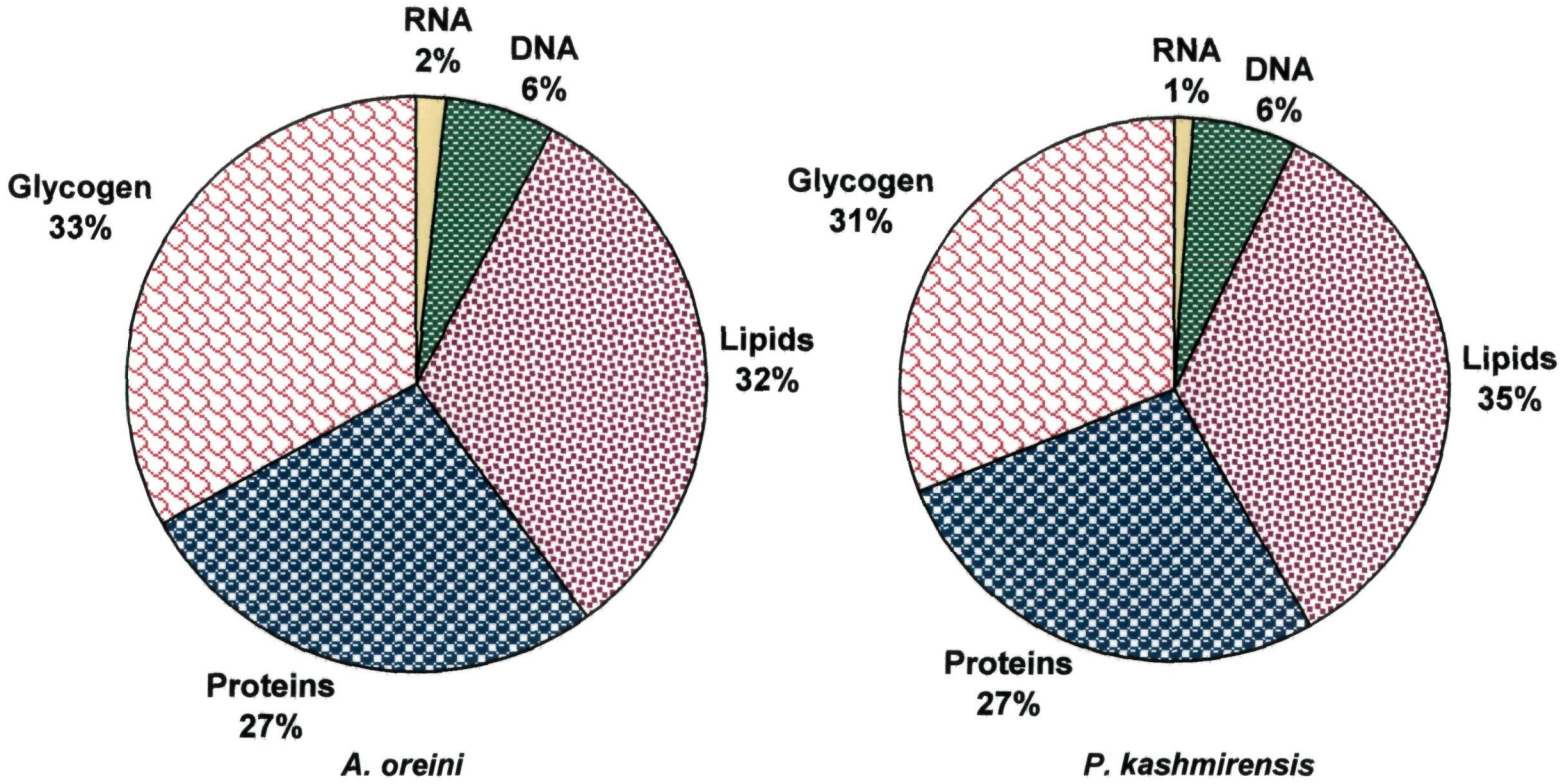


Fig.23. Major biochemical components of *A. oreini* and *P. kashmirensis* (360° assumed as 100%). Actual values are shown in Table 14.

the data between *A. oreini* and *P. kashmirensis* reveals that *P. kashmirensis* had significantly more glycogen, proteins, lipids, RNA and DNA than *A. oreini* (Table 14).

The results of SDS–PAGE revealed a heterogeneous polypeptide profile in the two parasites under study. A total of 16 and 14 polypeptides of different molecular weight were resolved in *A. oreini* and *P. kashmirensis*, respectively in coomassie brilliant blue stained gels. Further, the protein profile in male and female *P. kashmirensis* were also studied separately in order to find out similarity and differences between them. A total of 12 and 13 polypeptides were resolved in male and female parasites, respectively (Plate 6, Fig A). The apparent molecular weights of these polypeptides are presented in Table 15. Most of the polypeptides were found common in both male and female worms but there are 1 specific polypeptides in male and 2 in female *P. kashmirensis* (Plate 6 Fig. A; Fig. 24). The apparent molecular weight of these characteristic polypeptides are presented in Table 16.

It has been reported that CBB R–250 stain is less sensitive than the silver stain and many conjugated proteins are not stained with this dye therefore, silver staining of the gels was also performed which resolved a total of 30 and 26 polypeptides in whole homogenates of *A. oreini* and *P. kashmirensis*, respectively (Plate 6 Fig. B). Thus, some additional polypeptides were detected by silver staining which were absent in CBB R–250 stained gels (Figs. 25, 26, 27). The apparent molecular weights of the

Plate 6. The protein profiles of *A. oreini* (O) and *P. kashmirensis* (P ♂+♀) whole homogenates along with standard marker proteins (S) of known molecular weight by SDS- PAGE. The gels were stained with coomassie brilliant blue R-250 (A) and silver stain (B). The arrows indicate characteristic polypeptides present in female (P♀) and arrow heads in male (P♂) *P. kashmirensis*

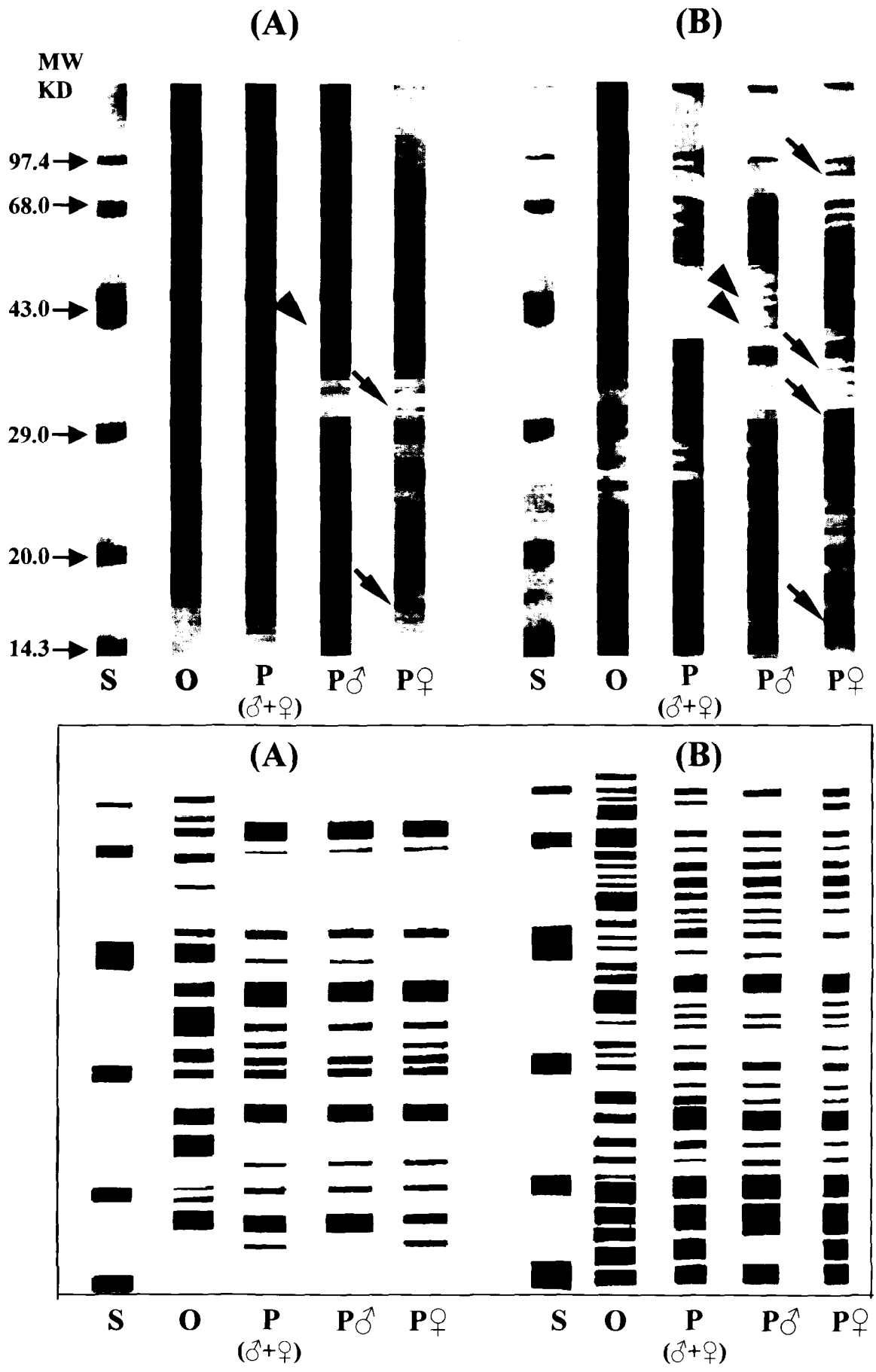


Plate 6

Table 15. Apparent molecular weight (in KD) of polypeptides resolved by CBBR- 250 staining procedure in *A. oreini* and *P. kashmirensis.*

A. oreini	*P. kashmirensis*		
Whole homogenate	Whole homogenate (male + females)	Male homogenate	Female homogenate
97.4	72.5	72.5	72.5
82.5	65.0	65.0	65.0
68.0	45.0	45.0	45.0
65.0	42.7	42.7	–
57.4	37.5	37.5	37.5
48.5	33.0	33.0	33.0
43.0	29.5	–	29.5
36.0	28.0	28.0	28.0
33.0	26.5	26.5	26.5
29.5	24.9	24.9	24.9
27.0	21.3	21.3	21.3
23.0	19.9	19.9	19.9
22.1	18.5	18.5	18.5
19.7	18.1	–	18.1
19.5			
18.5			
Total **16**	**14**	**12**	**13**

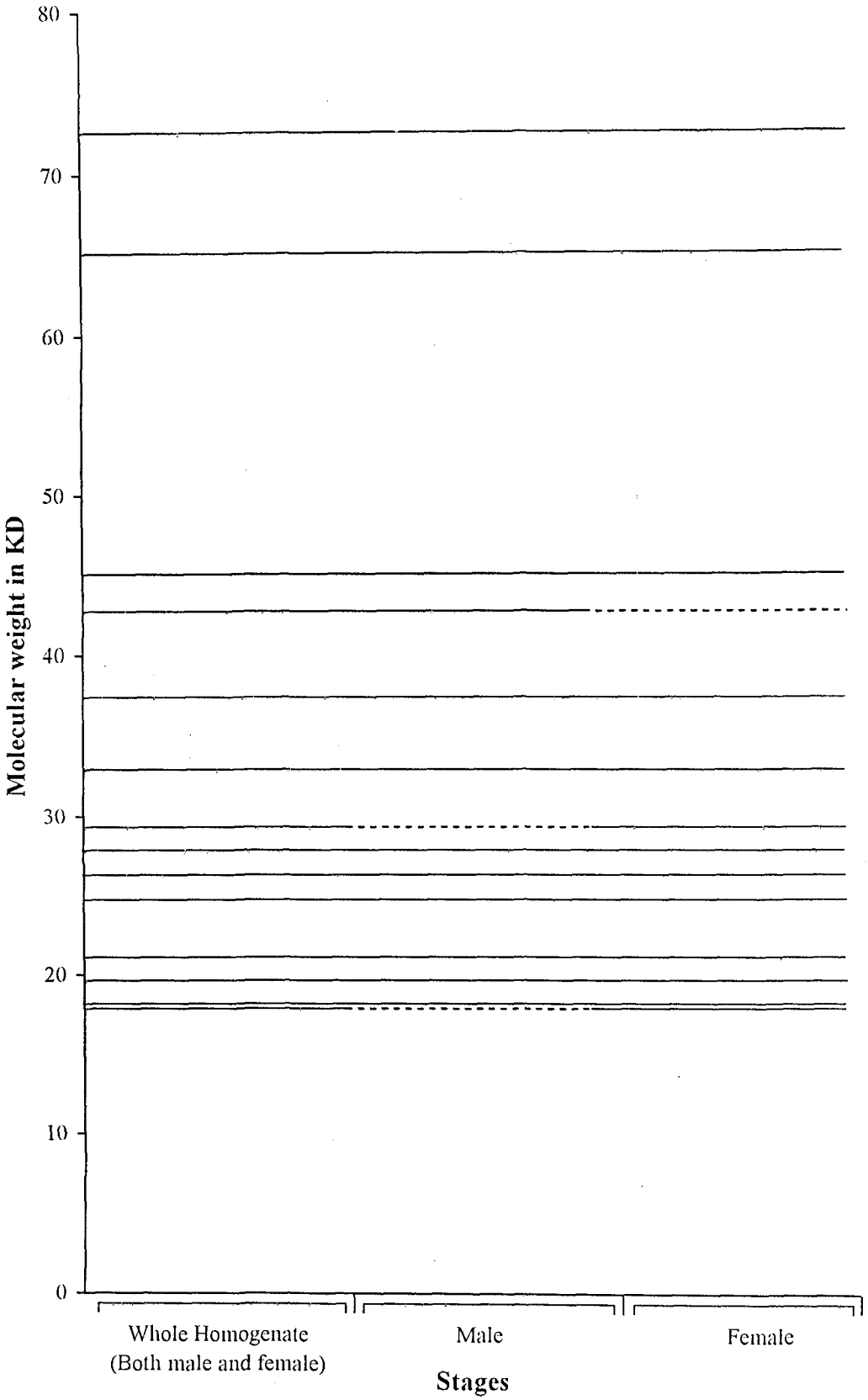


Fig. 24. Presence (——) and absence (------) of polypeptide in *P. kashmirensis* (Male, Female and Whole homogenate) by CBB-R250 staining procedure.

Table 16. Characteristic polypeptides with apparent molecular weight of the male and female *P. kashmirensis* by SDS-PAGE.

CBB-R 250 stained gel				Silver stained gel			
Male		Female		Male		Female	
No.	Mr. (in KD)	No.	Mr. (in KD)	No.	Mr. (in KD)	No.	Mr. (in KD)
1	42.7	–	–	–	–	1	86.7
–	–	1	29.5	1	47.0	–	–
–	–	1	18.1	1	42.7	–	–
				–	–	1	36.3
				–	–	1	29.5
				–	–	1	18.1

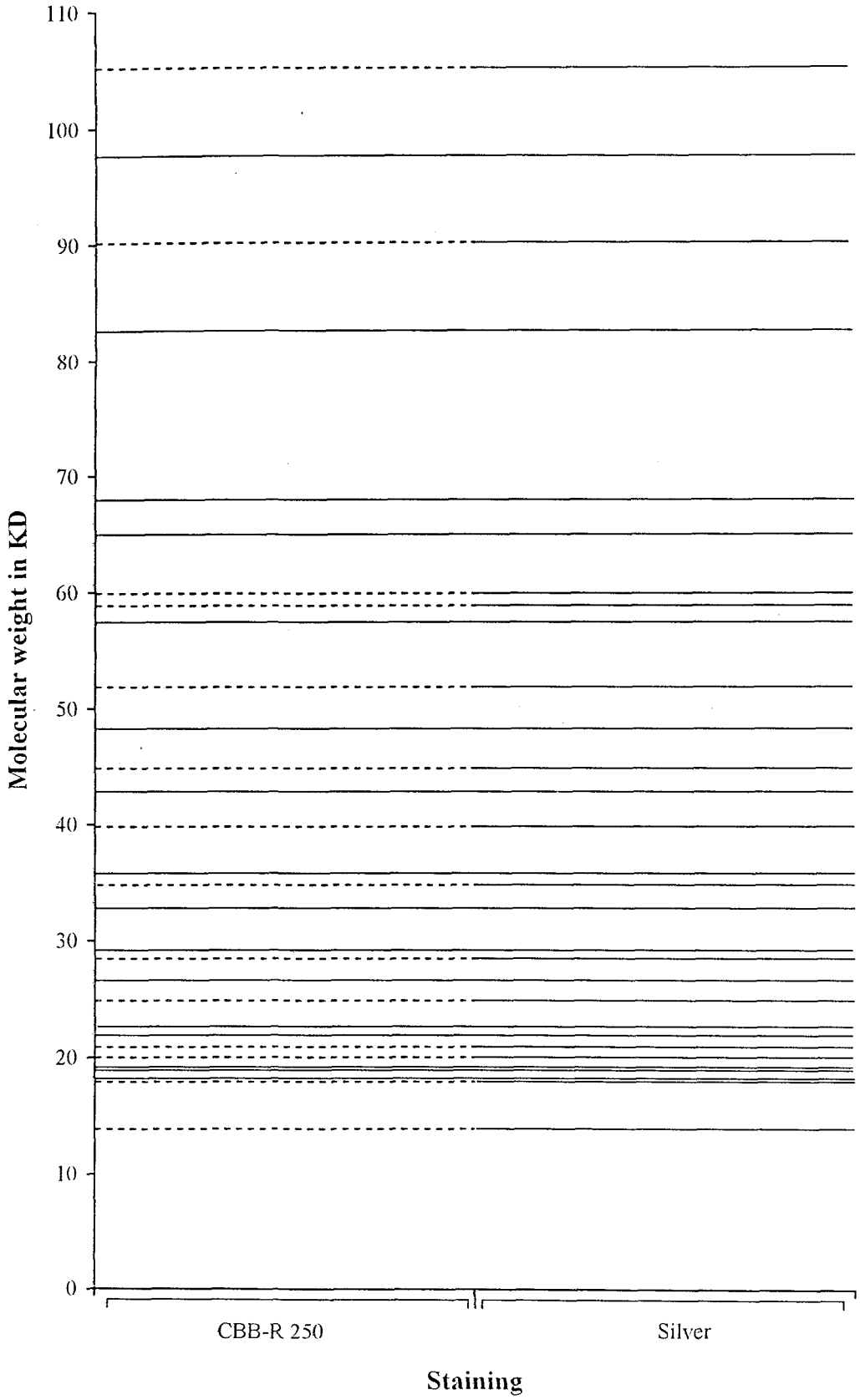


Fig. 25. Presence (——) and absence (------) of polypeptide in whole homogenate of *A. oreini* by CBB-R 250 and silver staining procedure.

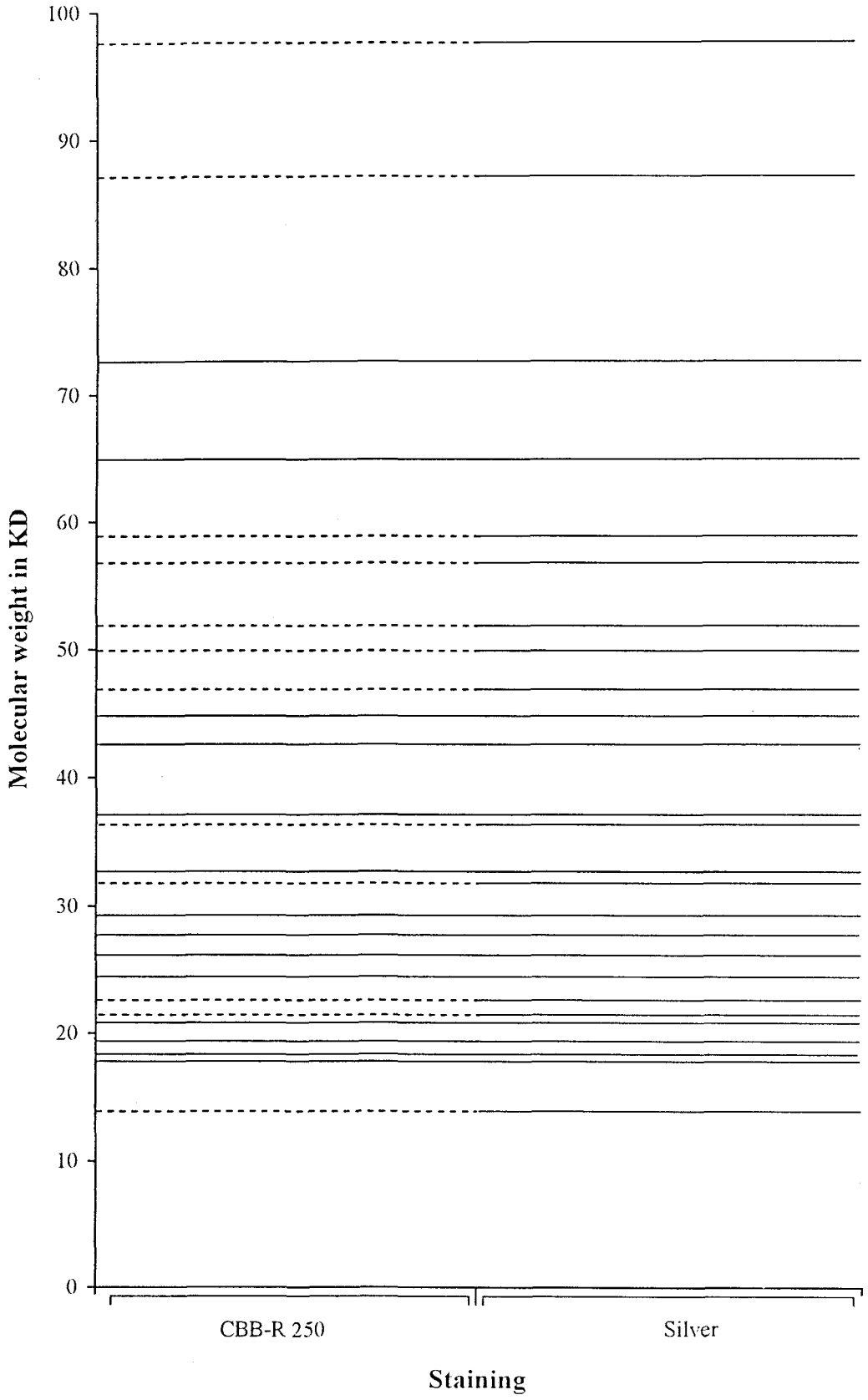


Fig. 26. Presence (——) and absence (------) of polypeptide in whole homogenate of *P. kashmirensis* by CBB-R 250 and silver staining procedure.

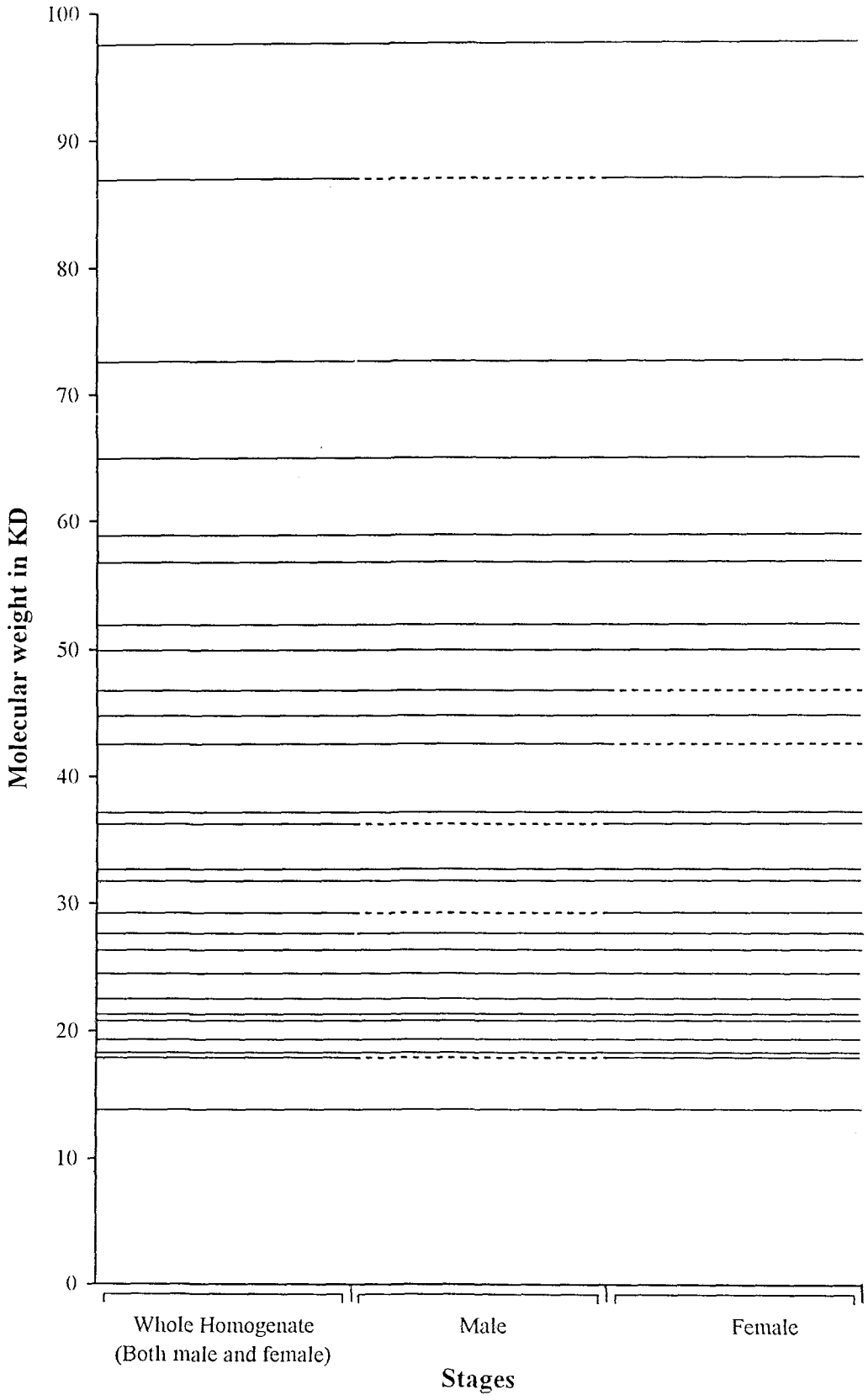


Fig. 27. Presence (——) and absence (------) of polypeptide in *P. kashmirensis* (Male, Female and Whole homogenate) by silver staining procedure.

polypeptides detected by silver staining are summarized in Table 17. It was observed that there are some specific polypeptides present in both male and female *P. kashmirensis*. In female 4 specific polypeptides of molecular weight 86.7 KD, 36.3 KD, 29.5 KD and 18.1 KD were observed while, in male 47 KD and 42.7 KD polypeptides were specific (Plate 6 Fig. B, Table 16).

It can be concluded from these results that *A. oreini* had comparatively more polypeptides than ***P. kashmirensis*** and there are some characteristic polypeptides in male and female ***P. kashmirensis***, which have different molecular weight thus, showing molecular heterogeneity in the protein profile.

[VI] Immunological Studies:

In order to study the antigenecity of *A. oreini* and *P. kashmirensis*, whole homogenates of both the parasites were used separately to raise the antibodies in rabbits. The raised antisera were tested to find out antigenicity of parasites and antibody titre by Ouchterlony's double diffusion and ELISA techniques, respectively.

[i] Ouchterlony's Double Diffusion Technique: The antigen–antibody reaction was carried out on agarose gel by double diffusion technique. The raised hyper–immune sera were found to react with respective antigen and a total of 2 and 4 distinct bands were found in *A. oreini* and *P. kashmirensis*, respectively, which indicate that there are more than one antigenic determinants in the parasite homogenates (Plate 7 Figs. C, D). When the

Table 17. Apparent molecular weight (in KD) of polypeptides resolved by silver staining procedure in *A. oreini* and *P. kashmirensis.*

A. oreini	*P. kashmirensis*		
Whole Homogenate	Whole Homogenate (male + female)	Male Homogenate	Female Homogenate
105.0	97.4	97.4	97.4
97.4	86.7	–	86.7
90.0	72.5	72.5	72.5
82.5	65.0	65.0	65.0
68.0	59.0	59.0	59.0
65.0	56.8	56.8	56.8
60.0	52.0	52.0	52.0
59.0	50.0	50.0	50.0
57.4	47.0	47.0	–
52.0	45.0	45.0	45.0
48.5	42.7	42.7	–
45.0	37.5	37.5	37.5
43.0	36.3	–	36.3
40.0	33.0	33.0	33.0
36.0	32.0	32.0	32.0
35.1	29.5	–	29.5
33.0	28.0	28.0	28.0
29.5	26.5	26.5	26.5
28.5	24.9	24.9	24.9
27.0	23.0	23.0	23.0
25.4	21.9	21.9	21.9
23.0	21.3	21.3	21.3
22.1	19.9	19.9	19.9
21.3	18.5	18.5	18.5
20.3	18.1	–	18.1
19.7	14.1	14.1	14.1
19.5			
18.5			
18.1			
14.1			
Total 30	**26**	**22**	**24**

Plate 7. Double immunodiffusion of *A. oreini* and *P. kashmirensis* antigens and antisera. A three wells pattern, 2 for antigens and one for hyper-immune antisera.

Figs.

A & B. Gel micrographs showing no reaction of antigens with control antisera (As).

C, D & E, F. Immunoprecipitate patterns of homologous (arrows) and heterologous reactions respectively, using the antisera raised against the antigens of ***A. oreini*** (Ag_1) and ***P. kashmirensis*** (Ag_2). Note the presence of precipitation arcs in homologous reaction and absence in heterologous reaction.

As_1 = antisera against ***A. oreini*** antigens (Ag_1).
As_2 = antisera against ***P. kashmirensis*** antigens (Ag_2).

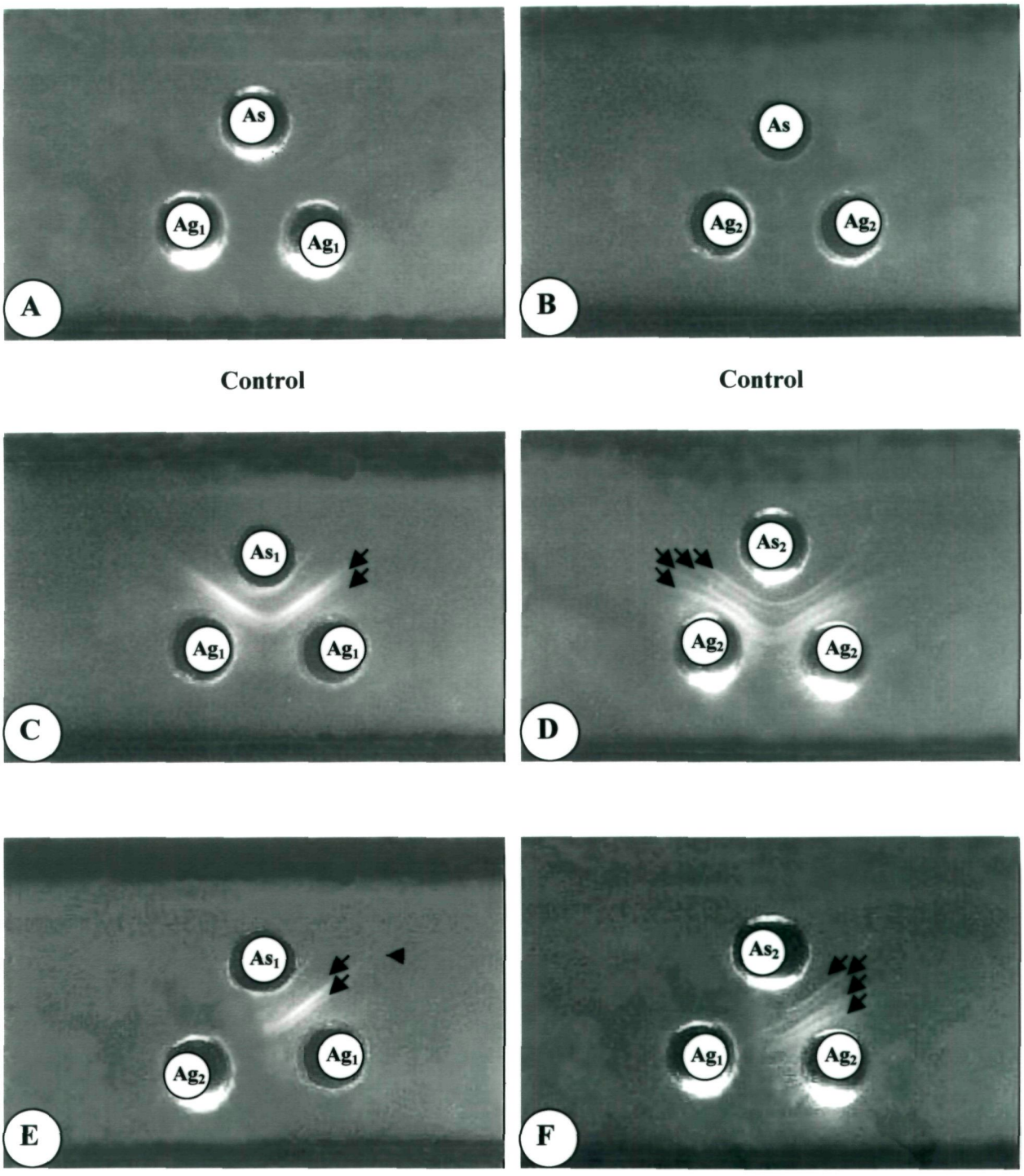

As
Ag1
Ag1
A
Control
As
Ag2
Ag2
B
Control
As1
Ag1
Ag1
C
As2
Ag2
Ag2
D
As1
Ag2
Ag1
E
As2
Ag1
Ag2
F

Plate 7

hyper–immune sera raised against *A. oreini* were tested against the antigens of *P. kashmirensis* and vice–versa, the cross reactivity between them was not observed (Plate 7 Figs. E, F). Similarly, the precipitation bands were not found when the antigens of both parasites were allowed to react with their respective naturally infected fish sera.

[ii] Enzyme Linked Immunosorbant Assay (ELISA): The immuno–diagnostic potential of the antigens of *A. oreini* and *P. kashmirensis* was tested by direct type of ELISA. A constant concentration (10 µg/ml) of the crude somatic antigen was coated onto the wells of the microtitre plate and was tested with the serially diluted hyper–immune sera. Under the present experimental conditions, absorbance values of 0.3 and 0.25 were established as the discriminating point for *A. oreini* and *P. kashmirensis* test sera, respectively. The cut–off point with the antisera raised against *A. oreini* and *P. kashmirensis* was found at a dilution of 1: 6400 and 1: 102400, respectively (Figs. 28, 29), indicating that the crude somatic homogenate of *P. kashmirensis* is more antigenic than *A. oreini*.

When the hyper–immune sera raised against the whole homogenate of *P. kashmirensis* were tested against the male and female antigens separately, the differences were observed in the antibody titre (Fig. 30). The cut–off point with antisera for female and male parasites was found at an absorbance of 0.24 and 0.27, respectively. The antigens of male and female parasites could be

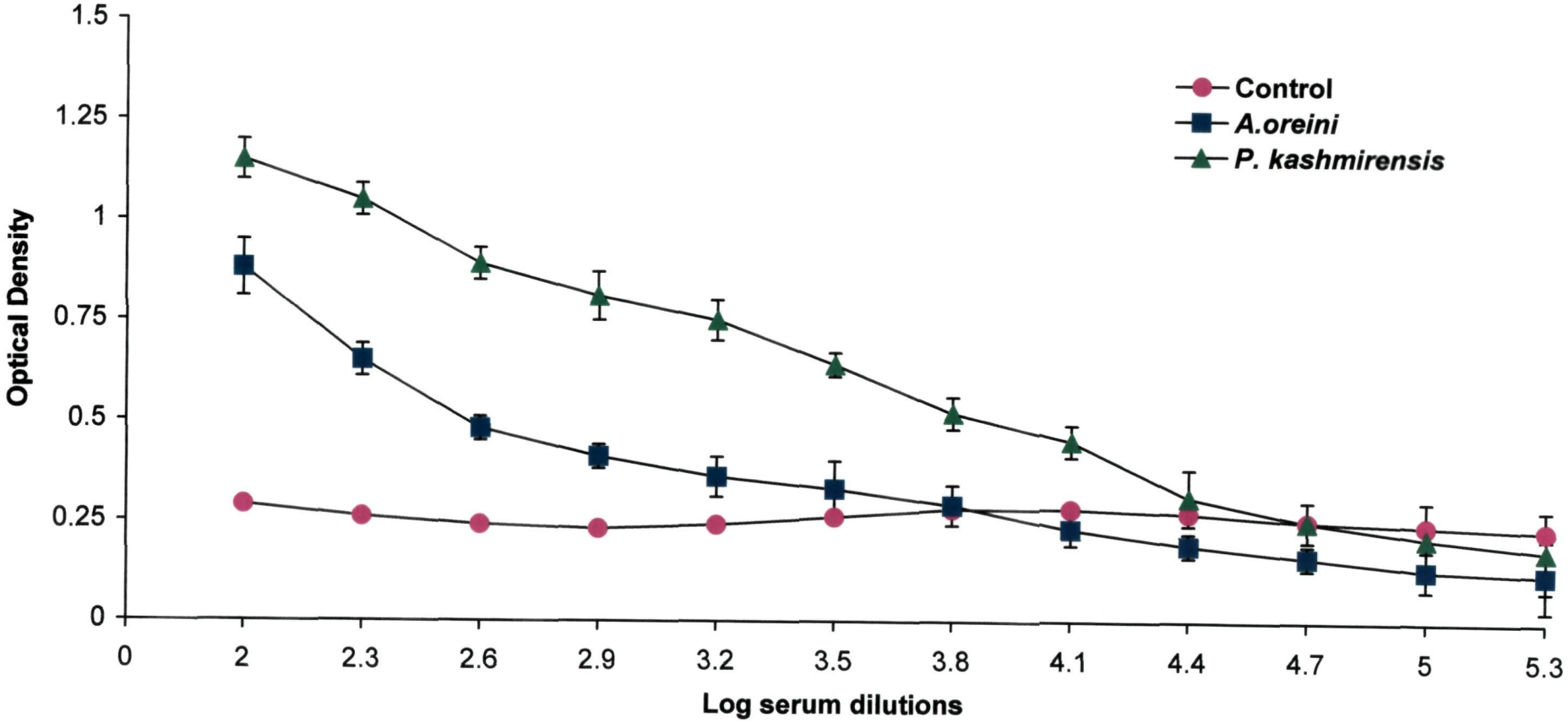


Fig. 28. Mean ELISA absorbance value for the raised antisera tested with crude antigens of *A. oreini* and *P. kashmirensis*. Vertical bar represents ± S.D.

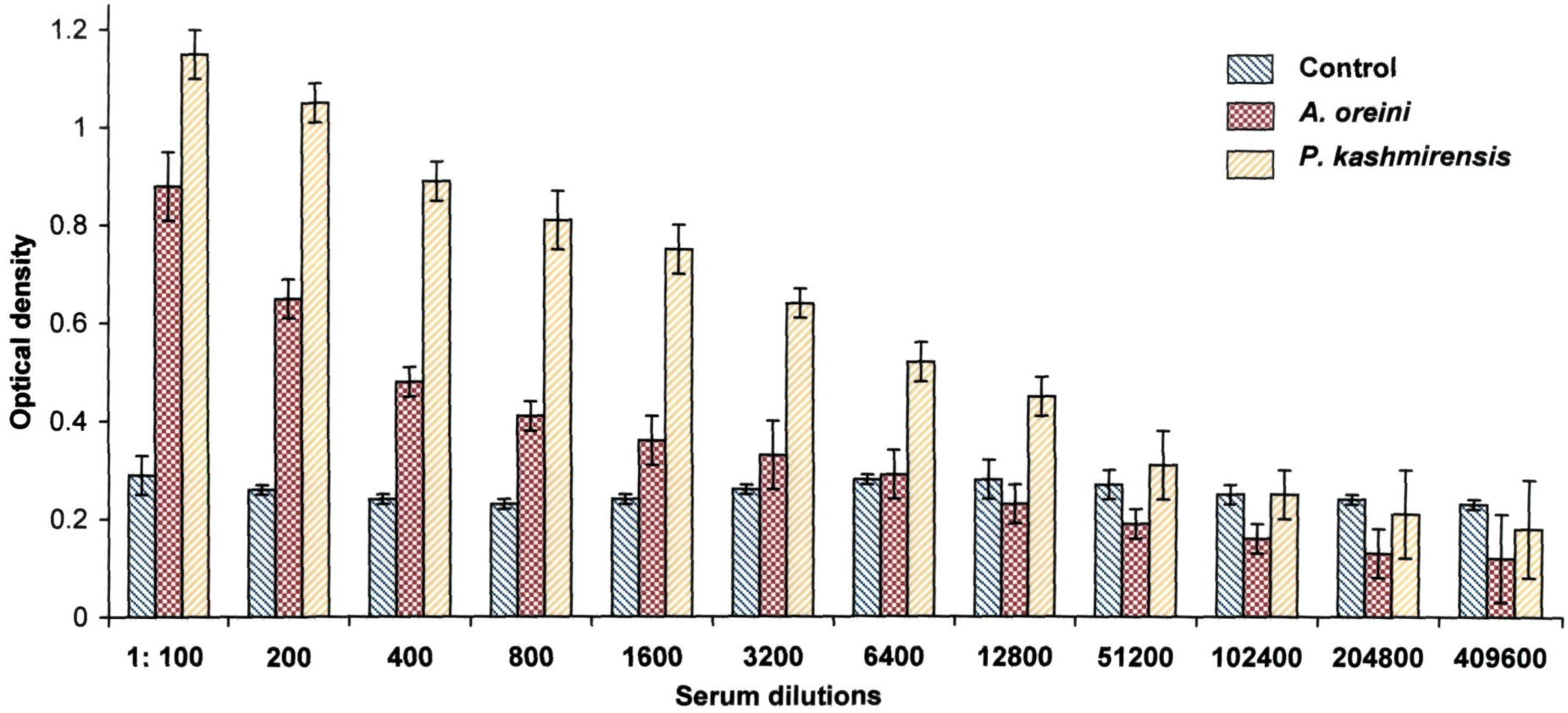


Fig.29. Absorbance value of the whole homogenates of *A. oreini* and *P. kashmirensis* as detected by the various dilutions of the test sera. The O.D. values for all the antigens tested is a mean of three different replicates.

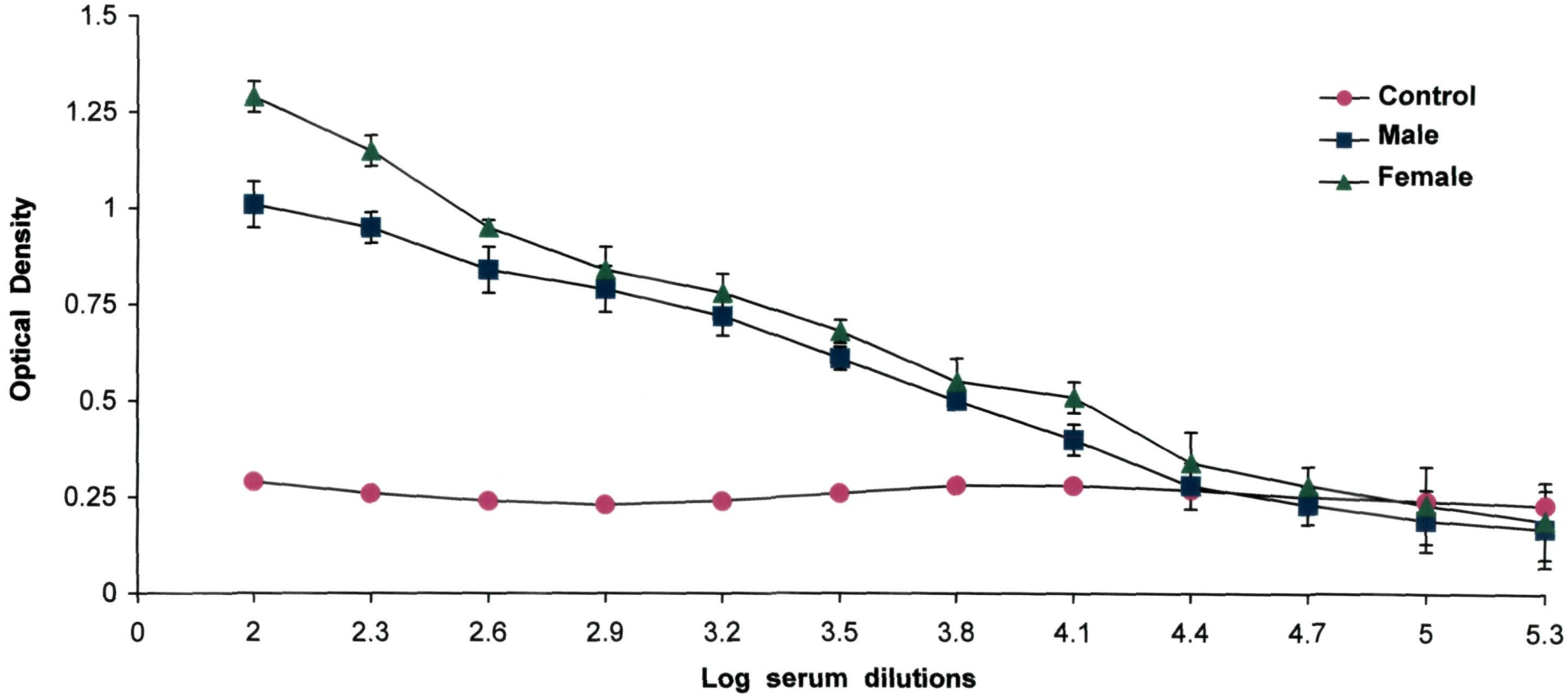


Fig.30. Mean ELISA absorbance values for the antisera raised against whole homogenate of *P. kashmirensis*, tested with the crude antigens of male and female parasites. Vertical bar represents ± S.D.

detected up to a dilution of 1:51200 and 1:204800, respectively (Fig. 31). These results indicate that female homogenate is more antigenic than male.

[VII] Topographical Effects of Drugs on *A. oreini* and *P. kashmirensis*:

The scanning electron microscopic studies on *A. oreini* and *P. kashmirensis* reveals some characteristic topographical features of different regions of parasites. *A. oreini* has smooth scolex and monozoic body plan which is characteristics of caryophyllidean cestodes. The scolex is undifferentiated from the remaining part of the body. The surface of the entire body is smooth having more or less same width (Plate 8, Figs. A–E). The body of *P. kashmirensis* is divided into three regions viz. proboscis, neck and trunk. The proboscis is long, cylindrical, having thorn–shaped hooks which are arranged in radial longitudinal rows and alternate in the adjacent rows. The proboscis is followed by an elongated cylindrical neck, which is dilated into balloon like bulb towards the proboscis end. The trunk is elongated, cylindrical and tapers towards the posterior end (Plate 9, Figs. A–D).

In total 5 drugs belonging to five different groups with 3 different concentrations (Table 1) were screened against *A. oreini* and *P. kashmirensis* and topographical damage induced by these drugs were studied by scanning electron microscopy (SEM). The effect of drugs was evaluated by comparing the results with that of control (Plates 8, 9, 10, 11). Almost all the drugs used in the present study damaged the body surface but the nature and degree of

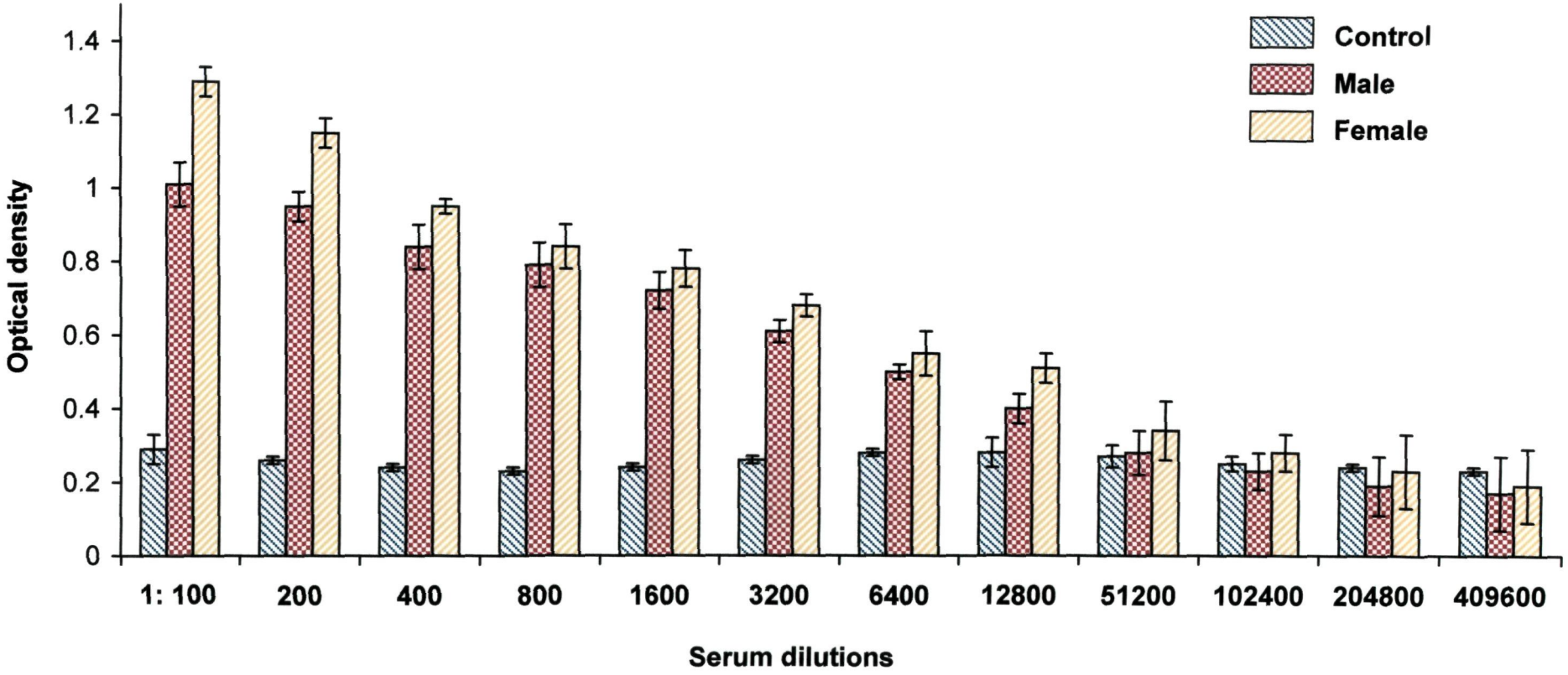


Fig.31. Absorbance value of the crude antigens of male and female *P. kashmirensis* as detected by the various dilutions of the test sera. The O.D. values for all the antigens tested is a mean of three different replicates.

Plate 8. Scanning electron micrographs of *A. oreini*, showing scolex (Figs. A, B, C, D) and body surface (Fig. F) at different magnifications. Note the absence of special attachment organs on the scolex.

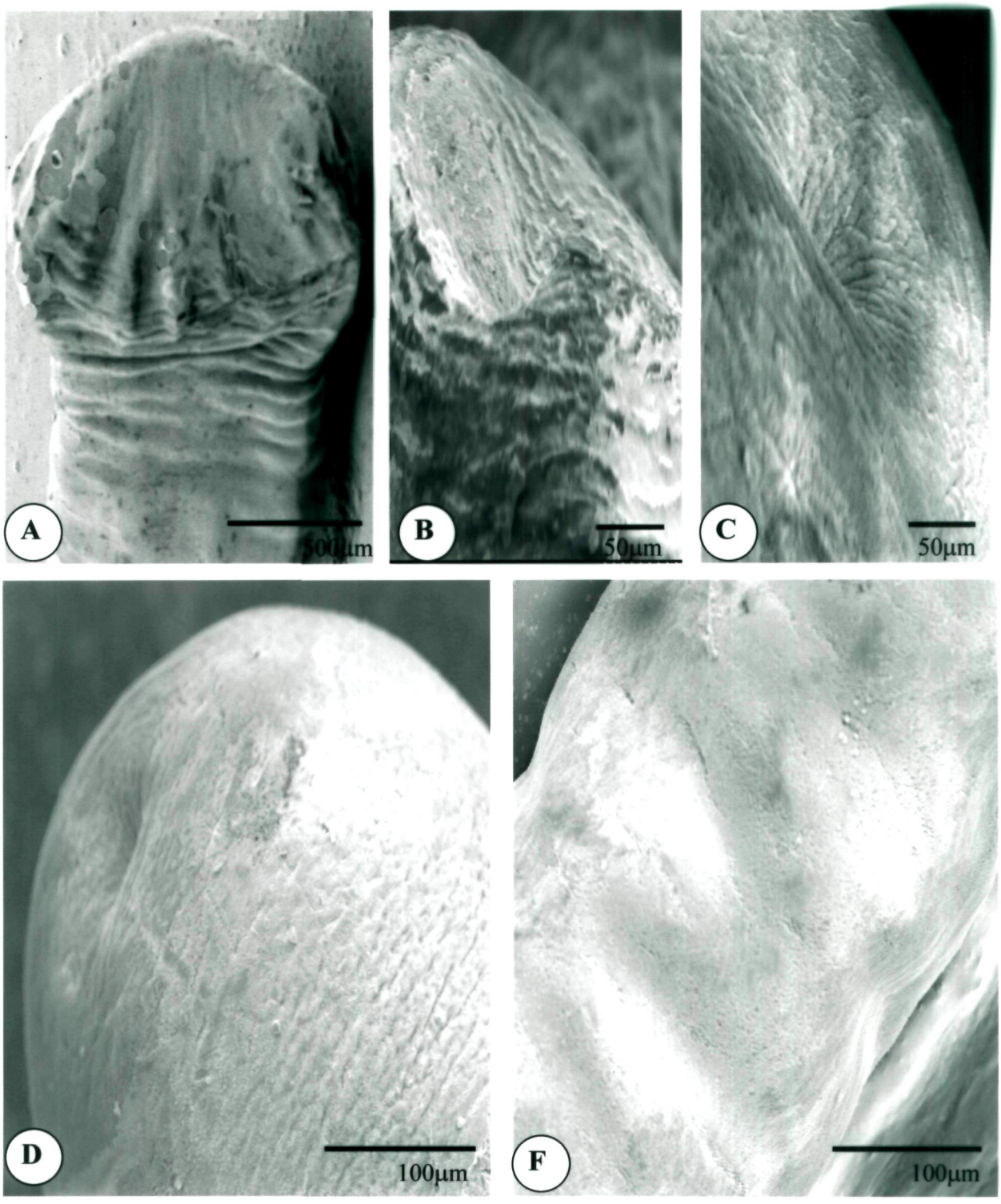


Plate 8

Plate 9. Scanning electron micrographs of *P. kashmirensis*, showing different body regions.

Figs.

A. Microphotograph showing the armed proboscis (arrow head) and bulb (arrow).

B & C. Microphotographs, showing hooks (H). Note the size and arrangement.

D. Microphotograph showing the trunk (T).

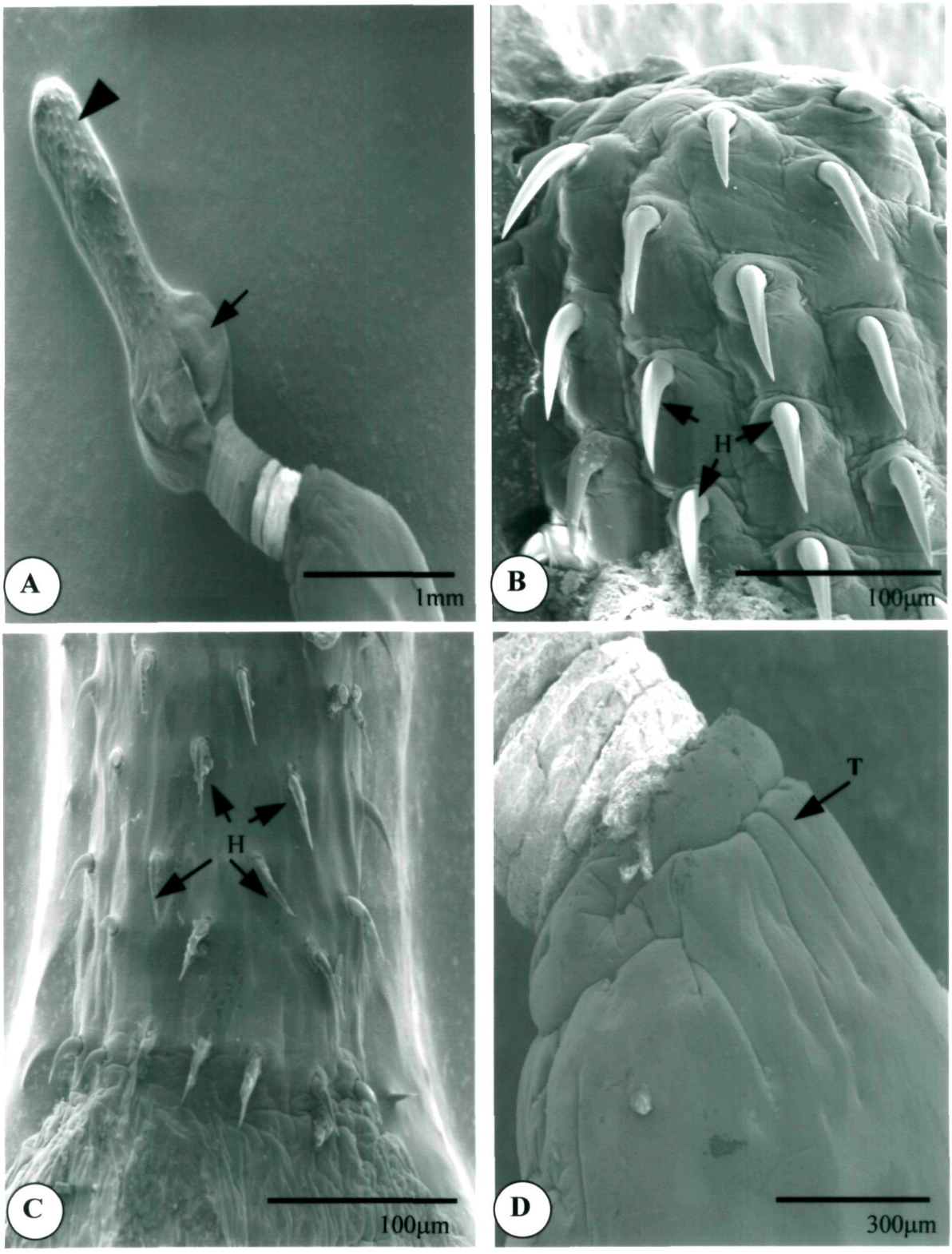
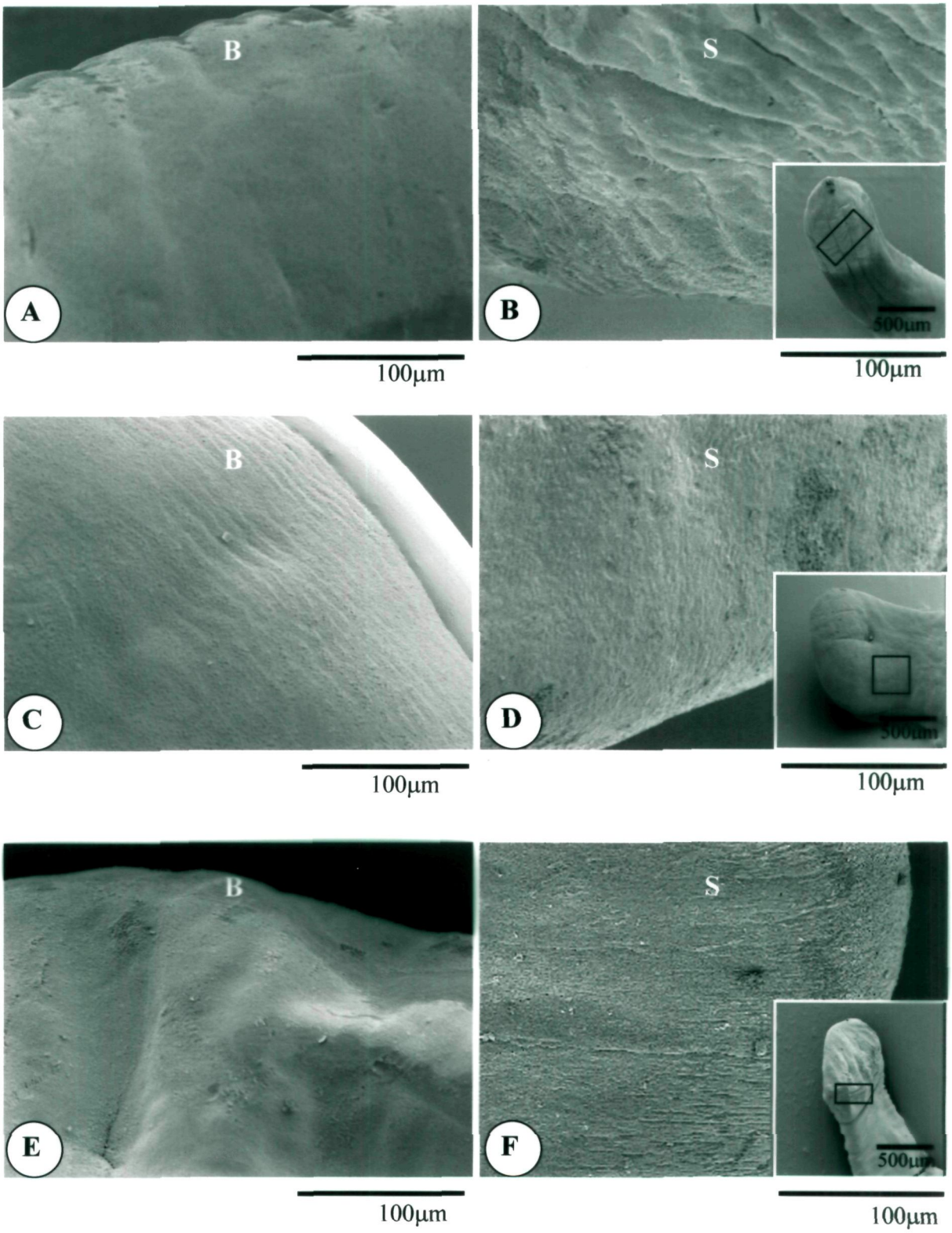


Plate 9

Plate 10. Scanning electron micrographs of scolex (S) and body surface (B) of untreated *A. oreini*, incubated in DMSO (Figs. A, B), ethanol (Figs. C, D) and double distilled water (Figs. E, F), showing surface structures. **Insert:** at higher magnification.


B
A
100µm
S
B
500µm
100µm
B
C
100µm
S
D
500µm
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B
E
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S
F
500µm
100µm

Plate 10

Plate 11. Scanning electron micrographs of proboscis (P) and trunk (T) of untreated *P. kashmirensis*, incubated in DMSO (Figs. A, B), ethanol (Figs. C, D) and double distilled water (Figs. E, F), showing the surface structures and hooks (arrows). **Insert:** at higher magnification.

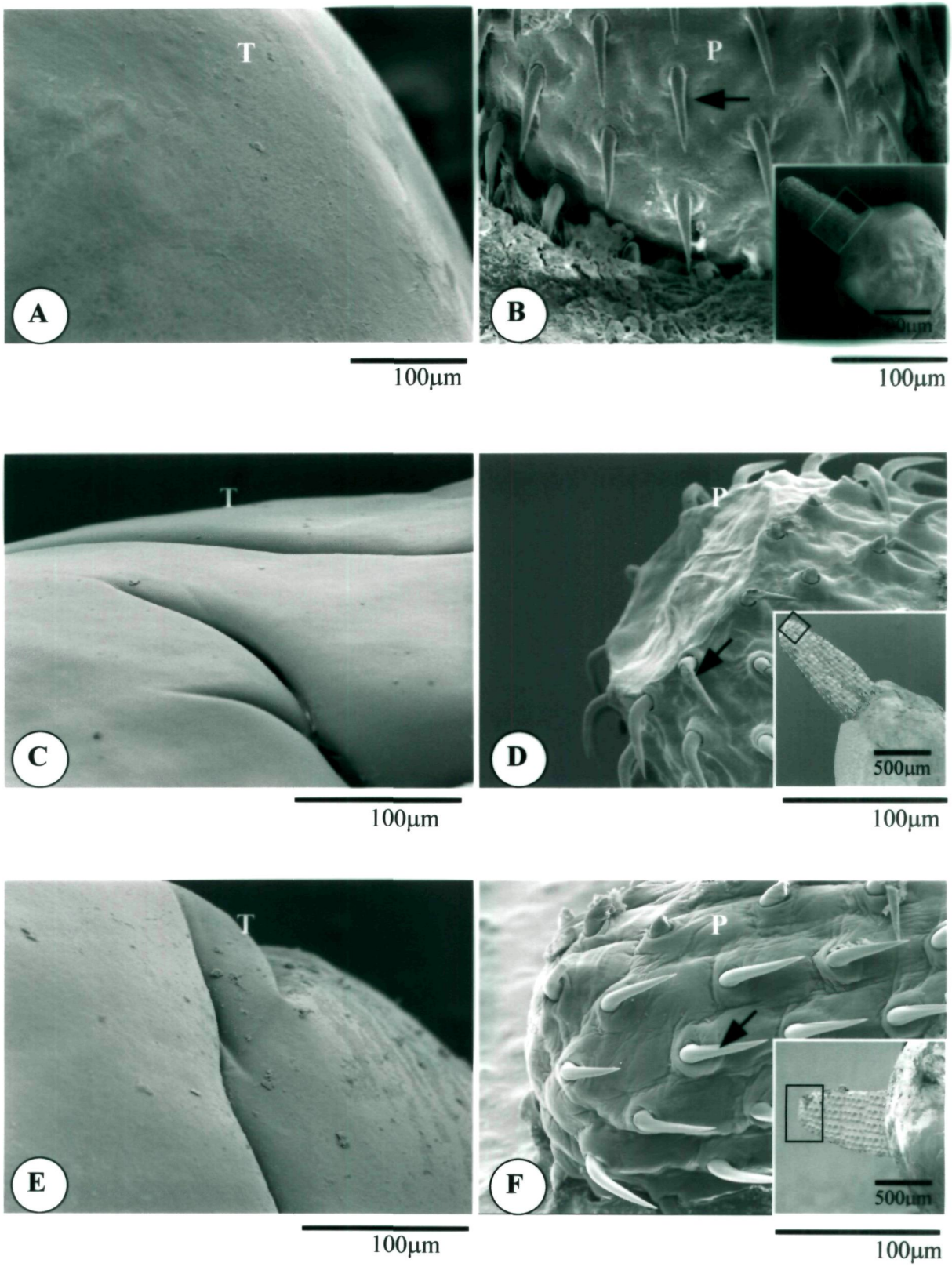

T
A
100µm
P
B
100µm
T
C
100µm
P
D
500µm
100µm
T
E
100µm
P
F
500µm
100µm

Plate 11

damage varied with the variation of groups and concentrations. Maximum damage was observed at 20 and 30 µmole concentrations in both parasites. All the drugs produced more pronounced effect on ***P. kashmirensis*** as compared to *A. oreini*.

[i] Mebendazole (Benzimidazole) : Mebendazole did not produce any apparent damage on *A. oreini*, however, deformation of the body like shrinkage and grooves were observed on the scolex and body surface at 20 and 30 µmole concentrations (Plate 12, Figs. C–F). At 10 µmole concentration no damage was noticed (Plate 12, Figs. A, B). In contrast to *A. oreini*, this drug caused more damage to ***P. kashmirensis*** where shrinkage of proboscis, peeling of tegument, retraction and damage of hooks and deeper lesions were observed at all used concentrations (Plate 13, Figs. A–F). The degree of damage increased with the increase of drug concentration.

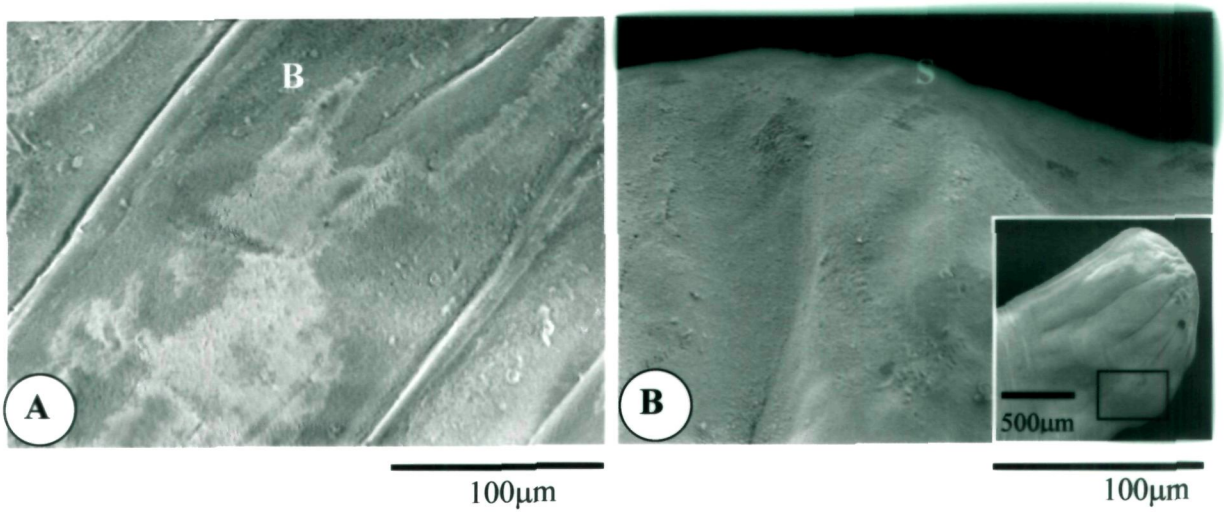
[ii] Rafoxanide (Salicylanilide) : Three different concentrations of rafoxanide were used, which caused retraction of proboscis and pits formation at 10 µmole concentration on ***P. kashmirensis*** whereas, almost no effect was noticed on *A. oreini* (Plates 14, 15, Figs. A, B). At 20 and 30 µmole concentrations, deeper grooves and cracks appeared on the scolex and body surface of *A. oreini* (Plate 14, Figs. C–F). More pronounced damages like peeling of tegument, coagulation of surface syncytium, retraction of proboscis, deeper pits on the trunk and bulb were observed on ***P. kashmirensis*** at these concentrations (Plate 15, Figs. C–F).

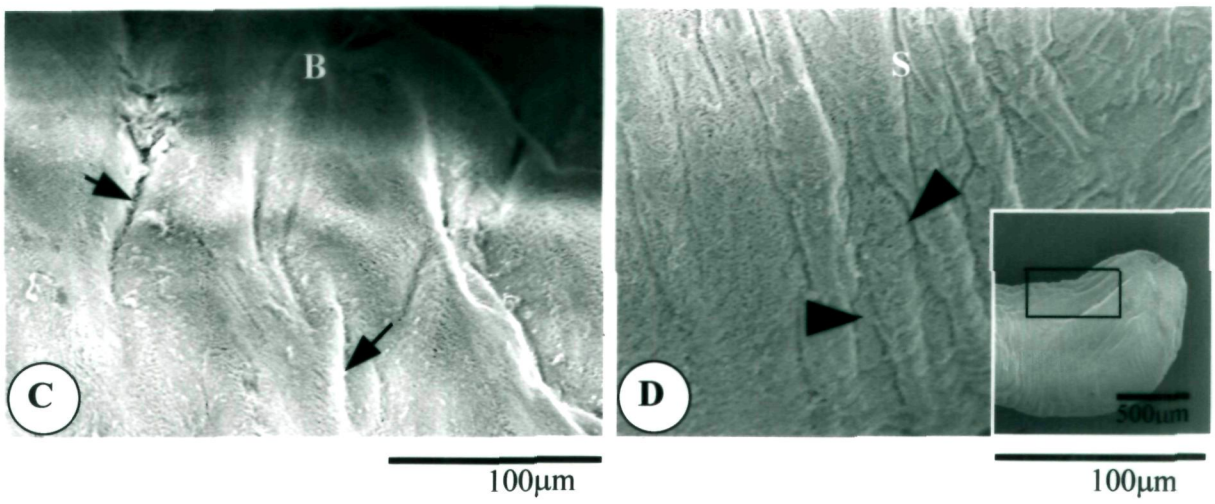
Plate 12. Scanning electron micrographs of scolex (S) and body surface (B) of *A. oreini*, treated with different concentrations of mebendazole.

Figs.

A & B. Effect of mebendazole at 10 µmole concentration, showing no apparent damage.

C, D & E, F. Effect of mebendazole at 20 and 30 µmole concentrations respectively, showing shrinkage and grooves on body surface (arrows) and scolex (arrow heads). **Insert:** at higher magnification.


B
A
100µm
S
B
500µm
100µm


B
C
100µm
S
D
500µm
100µm

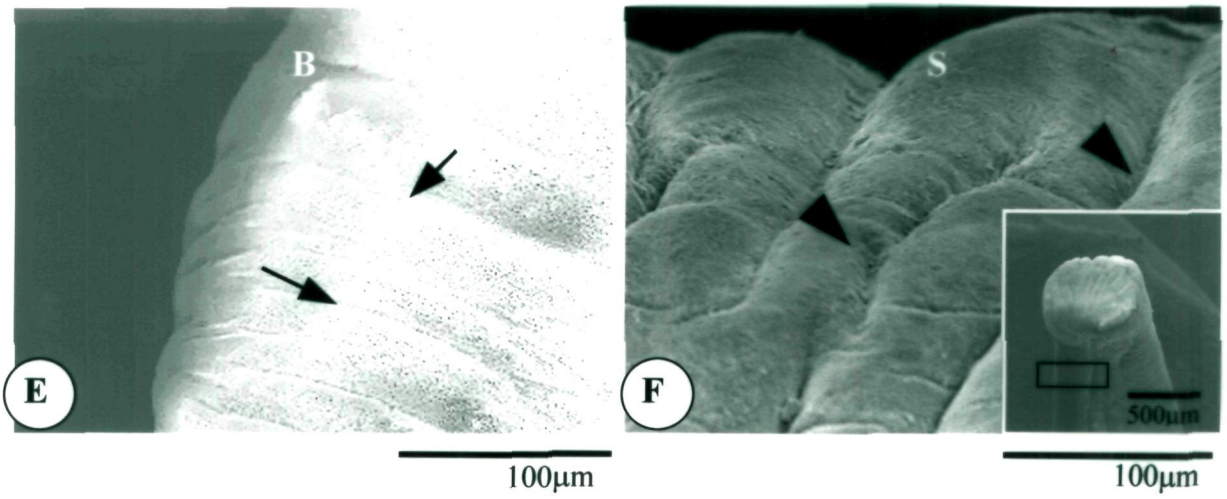

B
E
100µm
S
F
500µm
100µm

Plate 12

Plate 13. Scanning electron micrographs of proboscis (P) and trunk (T) of *P. kashmirensis*, treated with mebendazole at 10 (Figs. A, B), 20 (Figs. C, D) and 30 (Figs. E, F) µmole concentrations. Note the shrinkage (arrow heads), lesions, peeled off tegument and damage of hooks on proboscis (thick arrows) and body surface (thin arrow), which increased with the increase concentrations of drug. **Insert**: at higher magnification.

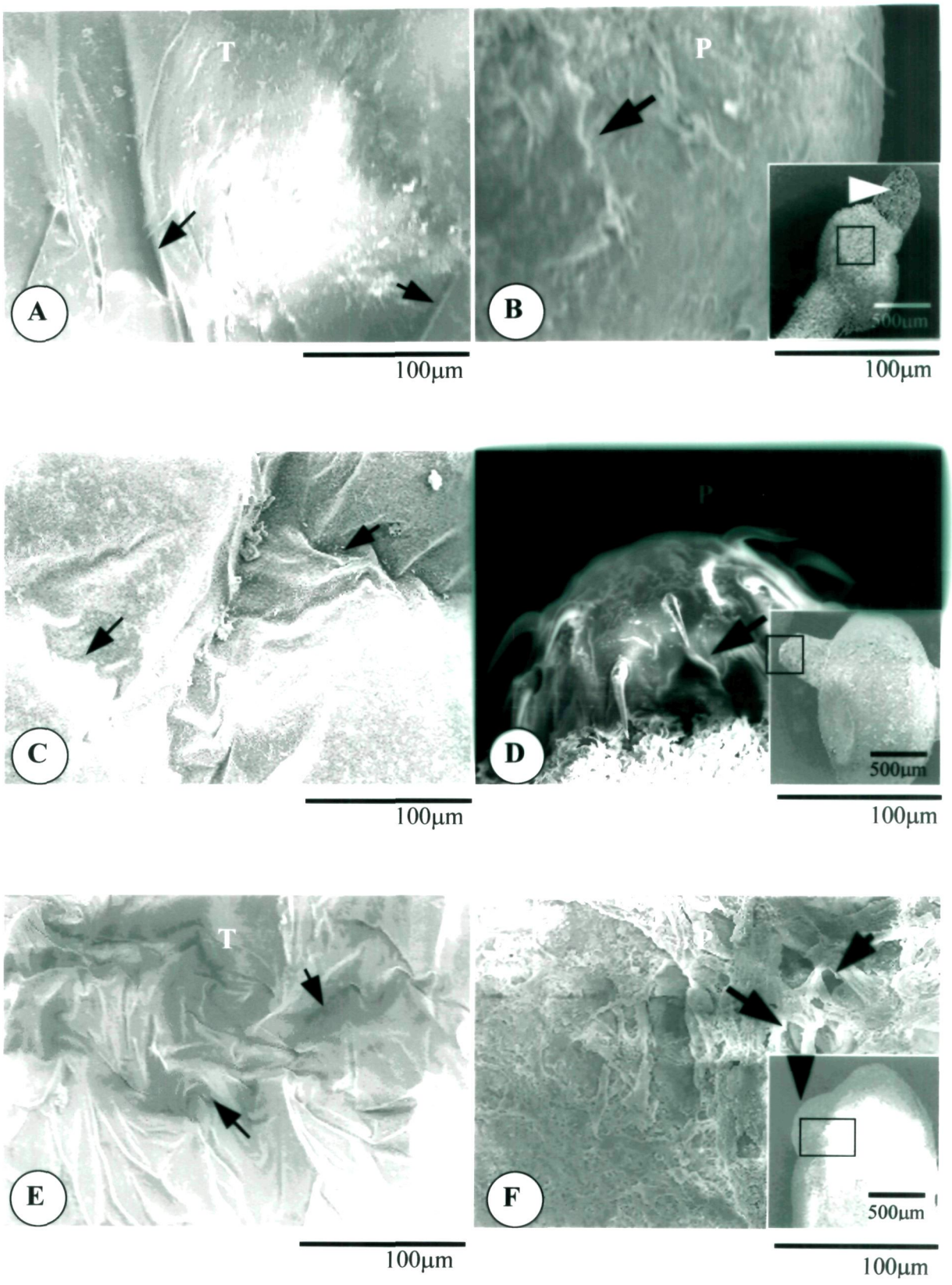

T
A
100µm
P
B
500µm
100µm
C
100µm
P
D
500µm
100µm
T
E
100µm
F
500µm
100µm

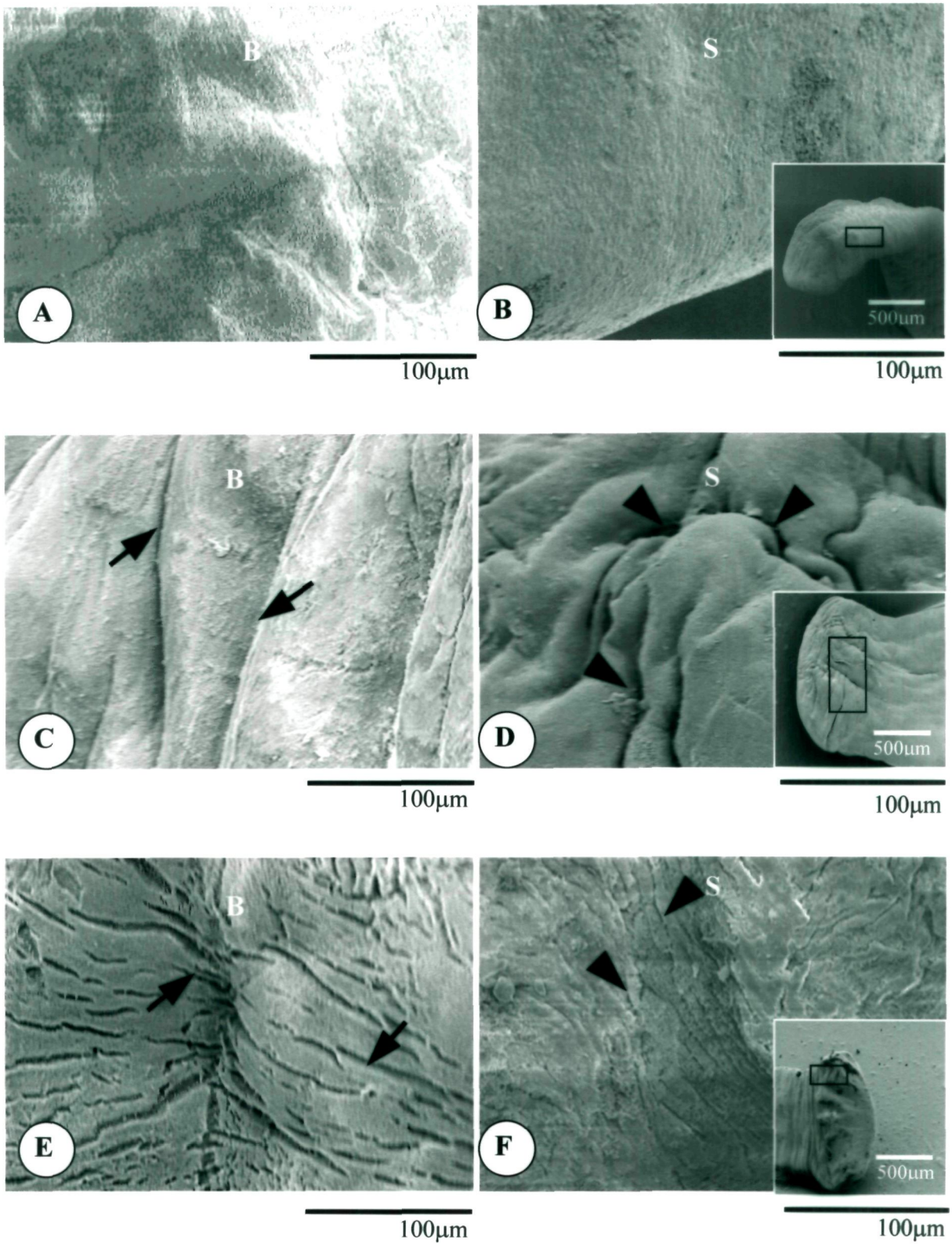
Plate 13

Plate 14. Scanning electron micrographs of scolex (S) and body surface (B) of *A. oreini*, treated with different concentrations of rafoxanide.

Figs.

A & B. Effect of rafoxanide at 10 µmole concentration, showing no apparent changes.

C, D & E, F. Effects of rafoxanide at 20 and 30 µmole concentrations respectively, showing grooves and cracks on scolex (arrow heads) and body surface (arrows). **Insert:** at higher magnification.


B
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100µm
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B
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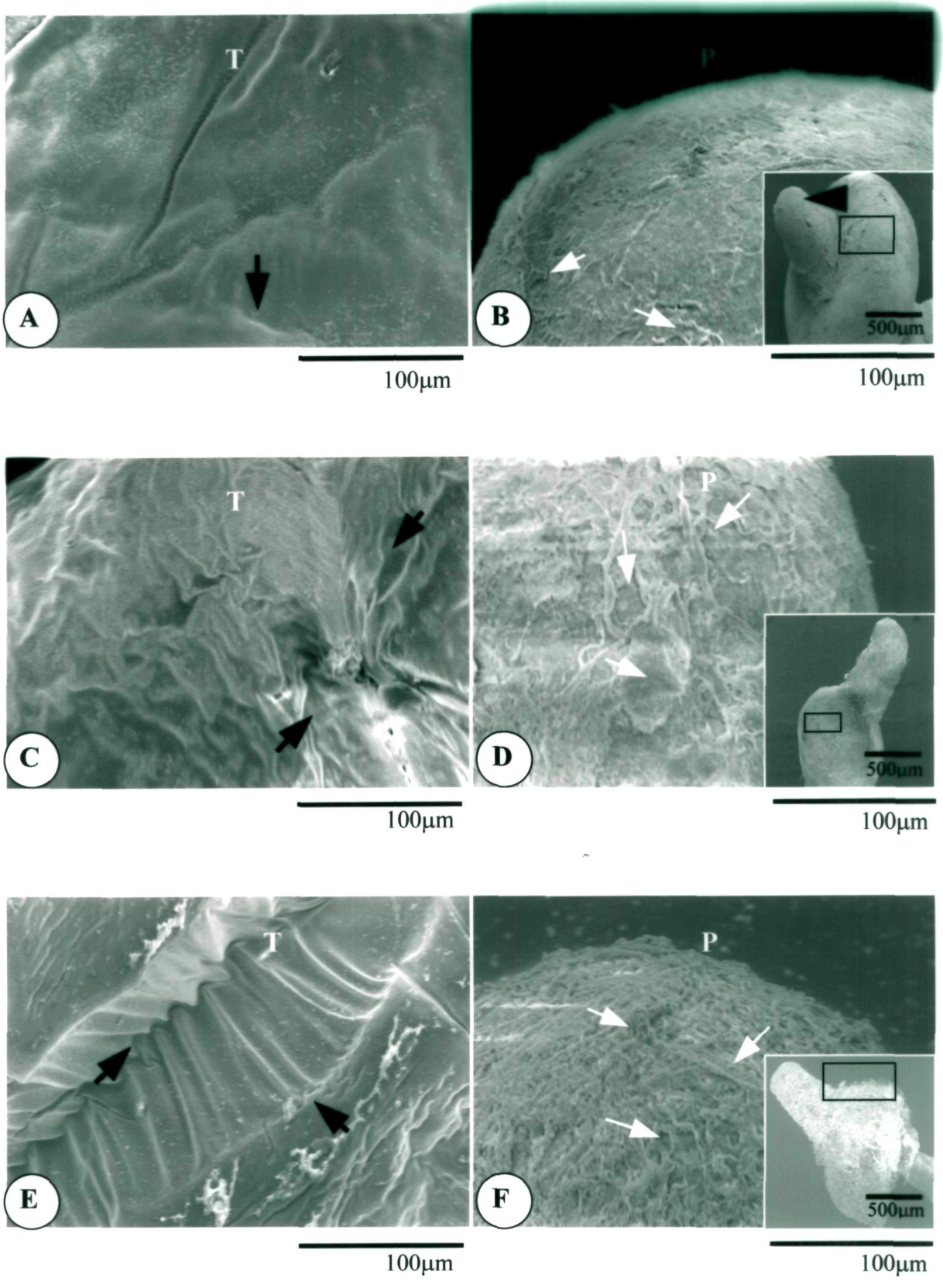
Plate 14

Plate 15. Scanning electron micrographs of proboscis (P) and trunk (T) of *P. kashmirensis*, treated with different concentrations of rafoxanide.

Figs.

A & B. Effect of rafoxanide at 10 µmole concentration showing retraction of proboscis (arrow head) and pits on bulb (small arrows).

C, D &E, F. Effects of rafoxanide at 20 and 30 µmole concentrations respectively showing the deep lesions, peeled off tegument and coagulation of surface syncytium on the bulb (white arrows) and body surface (arrows). **Insert:** at higher magnification.


T
A
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C
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100µm
T
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100µm
P
F
500µm
100µm

Plate 15

[iii] Nitroxinyl (Halogenated Phenols): The damage caused by the nitroxinyl varies with the concentrations of the drug and regions of the worms. This drug produced more effect on the scolex and proboscis at 10 and 20 µmole concentrations (Plates 16, 17, Figs. A–D) whereas, at 30 µmole concentrations the damage was more pronounced on the body surface (Plates 16, 17, Figs. E, F).

This drug at 10 and 20 µmole concentrations caused peeling of tegument on the scolex and grooves on the body surface of *A. oreini* (Plate 16, Figs. A–D), which became more deep and pronounced at 30 µmole concentration (Plate 16, Figs. E, F). In *P. kashmirensis*, appearance of deeper pits, deformation, damage and coagulation of surface syncytium was observed on the proboscis whereas, cracks and blister formation on the trunk due to this drug. The degree of damage increased with the increase concentration of drug (Plate 17, Figs. A–F).

[iv] Clorsulon (Sulphonamide) : This drug at 10 µmole concentration caused shrinkage of scolex and body surface of *A. oreini* and as a result deep pitted grooves appeared (Plate 18, Figs. A, B), which became more deep at 20 µmole concentration (Plate 18, Figs. C, D). In addition to this, peeling of tegument and deeper grooves were also observed on the scolex and body surface at 30 µmole concentration (Plate 18, Figs. E, F).

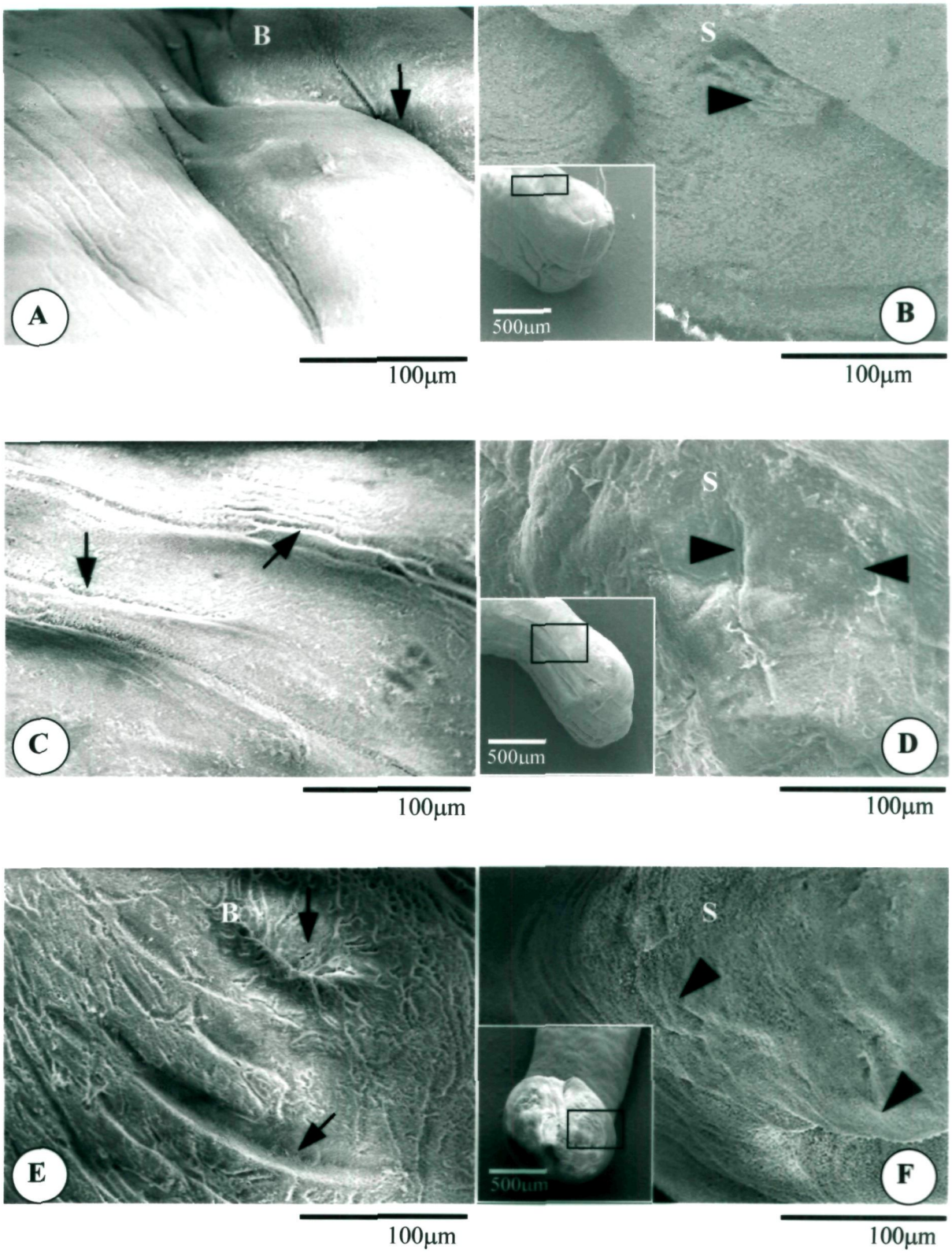
In *P. kashmirensis*, the damage caused by this drug was more pronounced than *A. oreini*, which include deformation and damage of hooks,

Plate 16. Scanning electron micrographs of scolex (S) and body surface (B) of *A. oreini*, treated with nitroxynil at three different concentrations.

Figs.

A, B & C, D. Effect of nitroxynil at 10 and 20 µmole concentrations respectively, showing peeled off tegument on scolex (arrow heads) and grooves on body surface (arrows).

E & F. Microphotographs showing the effect of drug at 30 µmole concentration, note the degree of damage on body surface (arrows) and scolex (arrow heads). **Insert:** at higher magnification.


B
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100µm
S
500µm
B
100µm
C
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S
500µm
D
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B
E
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S
500µm
F
100µm

Plate 16

Plate 17. Scanning electron micrographs of proboscis (P) and trunk (T) of *P. kashmirensis* treated with 10 (Figs. A, B), 20 (Figs. C, D) and 30 (Figs. E, F) µmole concentrations of nitroxynil, showing pits and coagulation of surface syncytium on proboscis (arrow heads) whereas, cracks and blisters formation on body surface (arrows). Note the degree of damage at different concentrations. **Insert:** at higher magnification.

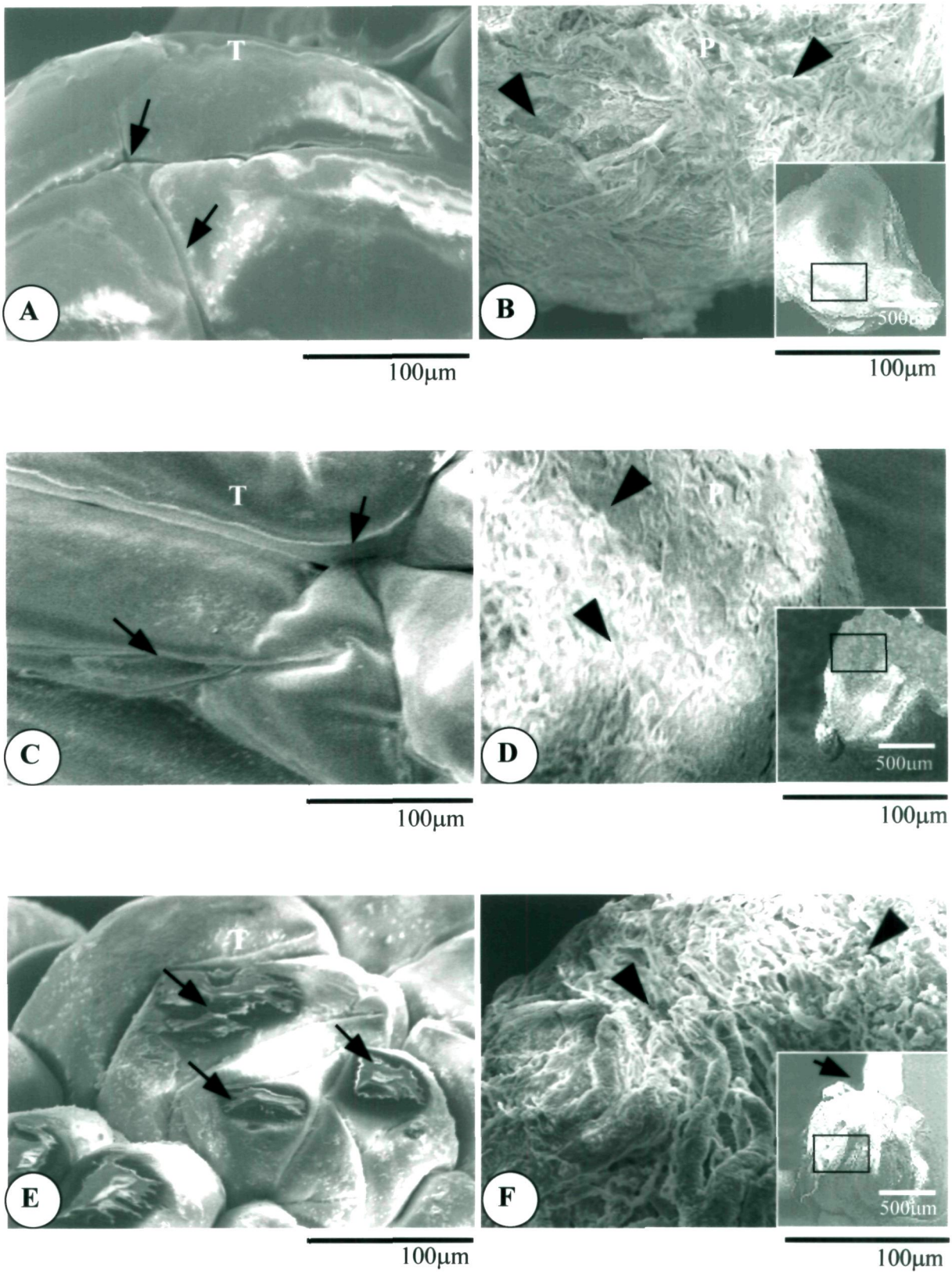

T
A
100µm
P
B
500µm
100µm
T
C
100µm
P
D
500µm
100µm
T
E
100µm
F
500µm
100µm

Plate 17

Plate 18. Scanning electron micrographs showing the effect of clorsulon on the scolex (S) and body surface (B) of *A. oreini* at 10 (Figs. A, B), 20 (Figs. C, D) and 30 (Figs. E, F) µmole concentrations.

Figs.

A & B. Microphotographs showing shrinkage and grooves on scolex (arrow heads) and body surface (arrows).

C & D. Microphotographs showing deeper grooves on scolex (arrow heads) and body surface (arrows).

E & F. Microphotographs showing peeled off tegument and deeper grooves on the scolex (arrow heads) and body surface (arrows). **Insert:** at higher magnification.

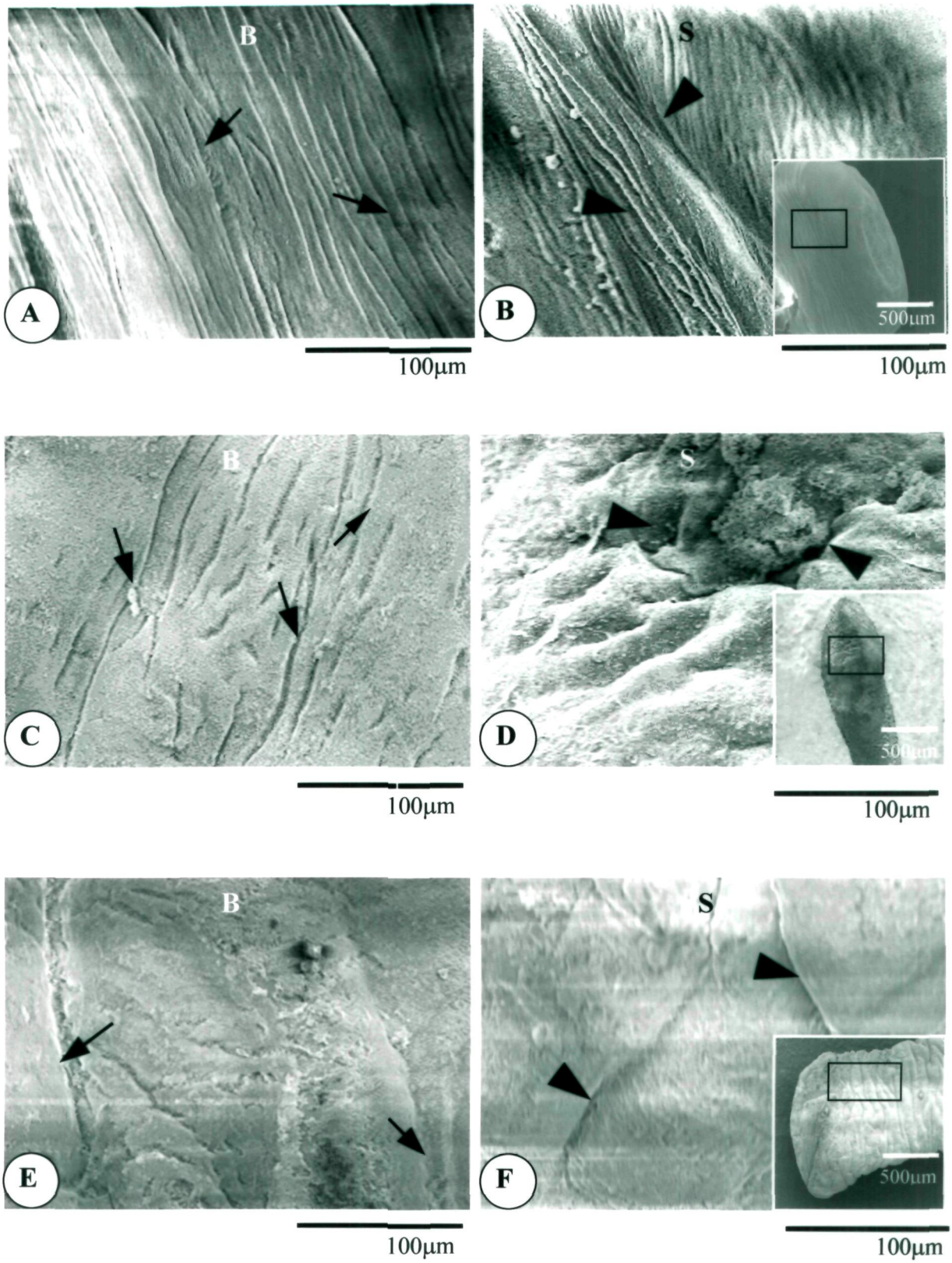

B
A
100µm
S
B
500µm
100µm
B
C
100µm
S
D
500µm
100µm
B
E
100µm
S
F
500µm
100µm

Plate 18

bulb and proboscis (Plate 19, Figs. B, D, F). However, cracks, deeper pits and peeling of tegument were observed on the trunk (Plate 19, Figs. A, C, E). This drug damaged the bulbous structure completely at 30 µmole concentration (Plate 19, Fig. F).

[v] Monensin (Ionophores) : Monensin at 10 µmole concentration produced more damage on the scolex and proboscis than body surface (Plates 20 and 21, Figs. A, B). At 20 and 30 µmole concentrations, deformation of scolex, shrinkage, lesions, peeling of tegument, blebbings and deeper pits were observed on ***A. oreini*** (Plate 20, Figs. C–F). In ***P. kashmirensis*** different kinds of changes were noticed on proboscis and trunk. This drug caused retraction of hooks, damage and shrinkage of proboscis and bulbous neck, peeling of tegument, shrinkage and blister formation on the trunk (Plate 21, Figs. A–F).

Thus it can be concluded that all the drugs tested, produced more or less same effects on both the parasites. Maximum effects were noticed by monensin on ***A. oreini*** whereas, by nitroxinyl on ***P. kashmirensis***. The actual damage on the topographical structure of ***A. oreini*** was observed in the following order monensin > rafoxanide > nitroxinyl > clorsulon > mebandazole whereas, on ***P. kashmirensis*** the order of effect was nitroxinyl > mebandazole > rafoxanide > monensin > clorsulon.

Plate 19. Scanning electron micrographs showing the effect of clorsulon on the proboscis (P) and trunk (T) of *P. kashmirensis* at 10 (Figs. A, B), 20 (Figs. C, D) and 30 (Figs. E, F) µmole concentrations.

Figs.

A, C & E. Microphotographs showing cracks (white arrows), deeper pits (thin arrows) and peeled off tegument on the body surface (thick arrows).

B, D & F. Microphotographs, showing deformation and damage of hooks and proboscis (arrow heads), bulb (white arrows). **Insert:** at higher magnification.

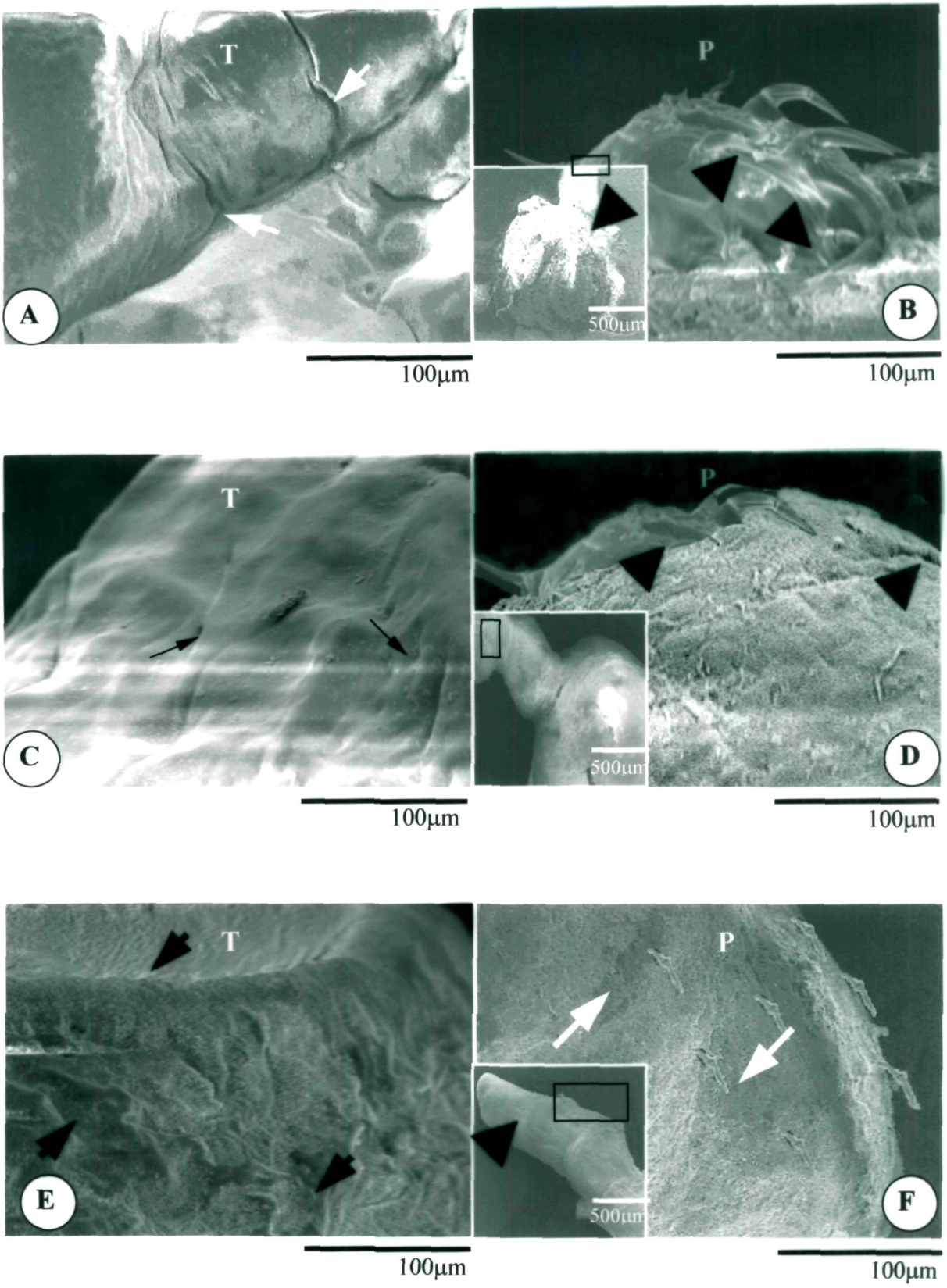

T
P
A
B
500µm
100µm
100µm
T
P
C
D
500µm
100µm
100µm
T
P
E
F
500µm
100µm
100µm

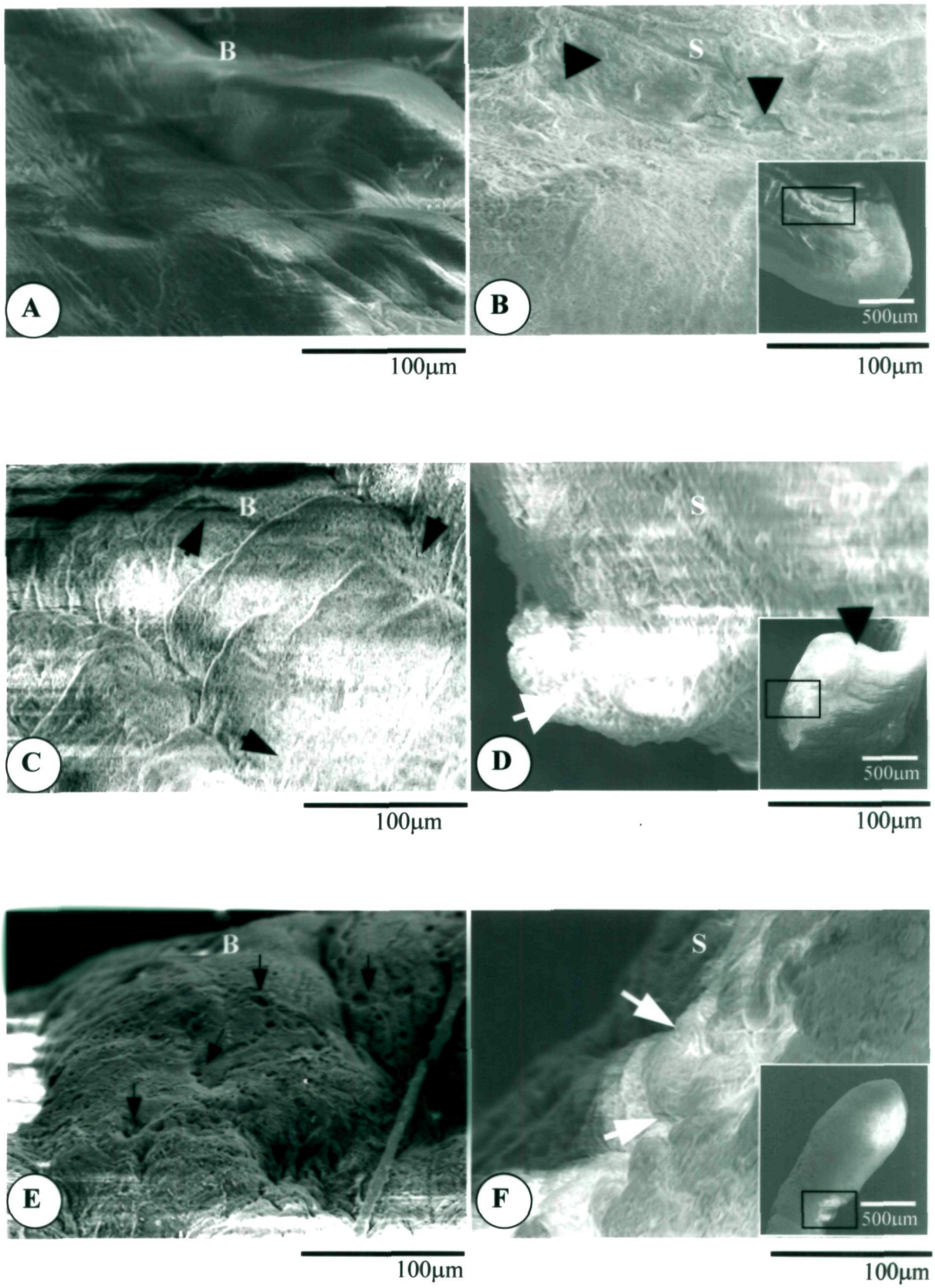
Plate 19

Plate 20. Scanning electron micrographs of scolex (S) and body surface (B) of *A. oreini,* treated with different concentrations of monensin.

Figs.

A & B. Effect of monensin at 10 µmole concentration, showing more damage on scolex (arrow heads) as compared to body surface.

C, D & E, F. Effect of monensin at 20 and 30 µmole concentrations respectively, showing blabbing on scolex (white arrows), peeled off tegument (arrows) and deeper pits (small arrows) on the body surface. **Insert:** at higher magnification.


B
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100µm
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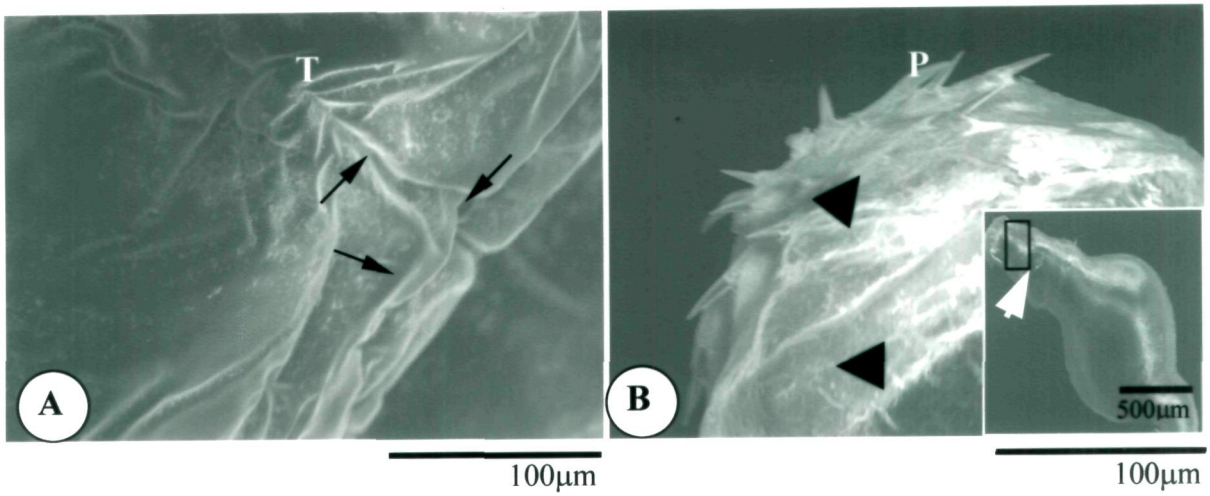
Plate 20

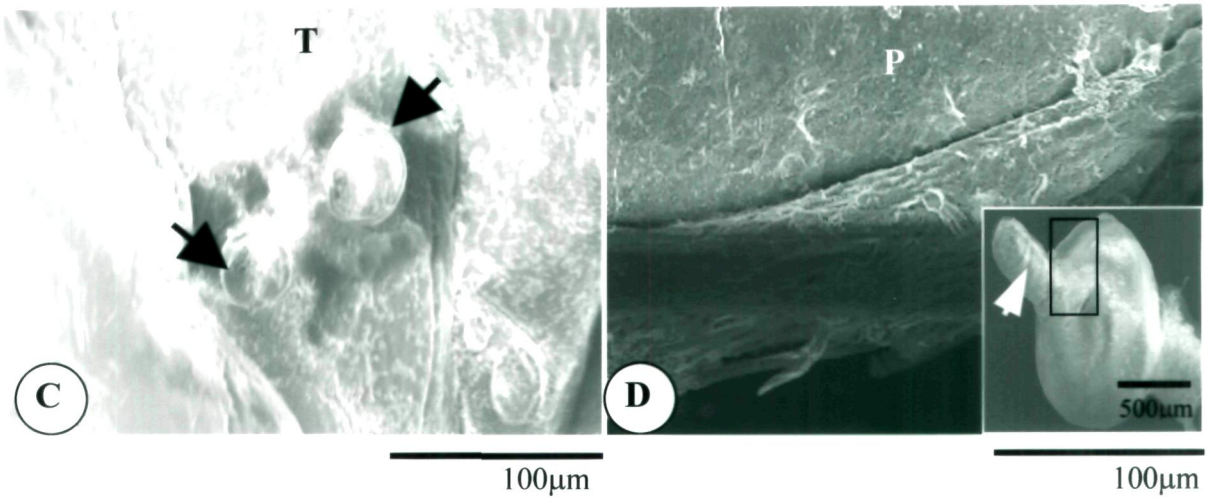
Plate 21. Scanning electron micrographs, showing, the effect of monensin, on proboscis (P) and trunk (T) of *P. kashmirensis*.

Figs.

A, C & E. Microphotographs showing peeled of tegument (small arrows), blisters (arrows) and shrinkage (white arrows) on body surface at 10, 20 and 30 µmole concentrations respectively.

B, D & F. Microphotographs showing damage of hooks and proboscis (arrow heads), shrinkage of proboscis (white arrows) and damage on bulb (arrows) at 10, 20 and 30 µmole concentrations respectively. **Insert:** at higher magnification.


T
A
100µm
P
B
500µm
100µm


T
C
100µm
P
D
500µm
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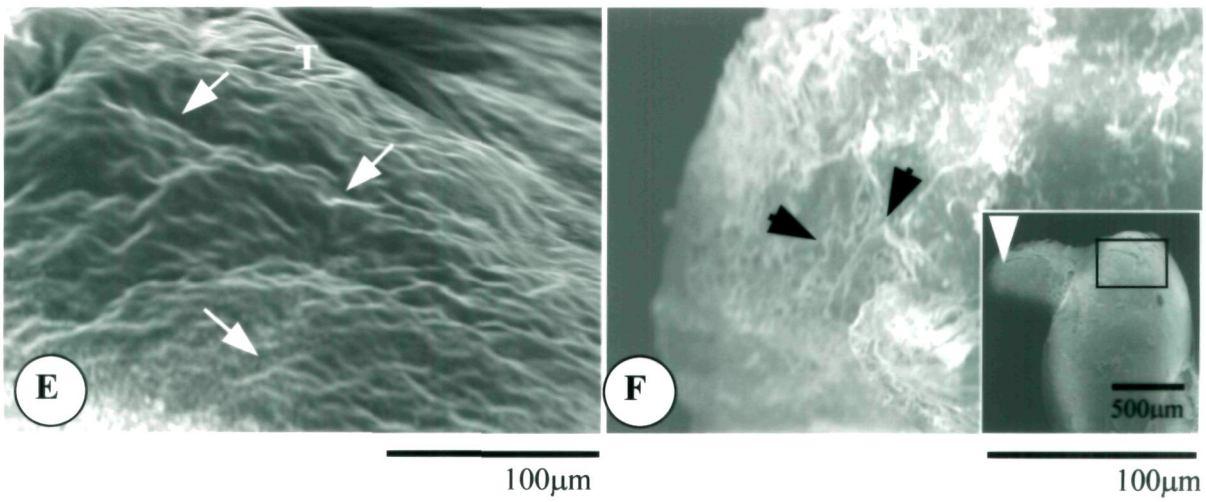
T
E
100µm
F
500µm
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Plate 21

DISCUSSION

DISCUSSION

[I] Prevalence of *A. oreini* and *P. kashmirensis* in the fishes of Kashmir:

Both ***A. oreini*** and ***P. kashmirensis*** were quite prevalent in the fishes of Kashmir. A total 28.44% fishes were found infected with these parasites, among which almost equal number of fishes harbour ***A. oreini*** (12.27%) and ***P. kashmirensis*** (12.07%). In contrast to this, low prevalence of these parasites has been reported by many workers **(Chishti and Peerzada, 1998; Khan *et al.*, 2004)**. Such differences may either be due to sample size or due to variation of habitat (running and standing water bodies). The present work has been carried out in running (lotic) whereas, the work published by above cited workers was from standing (lentic) water bodies and they had examined smaller number of fishes. The high prevalence in the lotic habitat (present study) may also be due to higher number of flora and fauna, as lotic habitat has comparatively more flora and fauna than lentic habitat **(Engblem and Lingdell, 1999)**. Similarly, **Amin (1987b)** reported that the fishes were frequently and heavily infected with ***P. bulbocolli*** in the river-connected and more eutrophic Tichigan Lake than in the landlocked Silver Lake and correlated it with the composition of invertebrate fauna accommodated at these sites.

Further, the prevalence of both parasites varies with the sites of fish collection. Maximum infection of ***A. oreini*** and ***P. kashmirensis*** was found in

Srinagar and Sopore respectively whereas, low prevalence of both parasites was found in Anantnag and Baramullah. Such differences can be correlated with the population of intermediate host at different sites and preference of diet by the definitive host. As it was suggested that the fishes give more preference to feed upon *Gammarus* (intermediate host of ***P. kashmirensis***) as compared to *Tubifex* (intermediate host of ***A. oreini***) and the population of *Tubifex* was higher in Srinagar due to more water pollution and low flow rate **(Anon, 2000)**. Similar suggestions have also been made by many workers on other helminth parasites **(Chubb, 1964a, b; Kochva, 1967; Kennedy, 1972a; Amin, 1975; 1987b; Munro *et al.*, 1989; Belghyti *et al.*, 1994)**. **Muzzall (1982)** showed a direct relationship between the size, seasonality and developmental state of *G. pseudolimnaeus* (intermediate host) and infections in the definitive fish host. The *Schizothorax* species consume about 30-35% aquatic invertebrates in its diet **(Jan and Das, 1970; Sulba and Das, 1970)** and thus the consumption of diet may be a determining factor for the prevalence of parasites in fishes.

All the four species of *Schizothorax* were found infected with both parasites and maximum infection was noticed in *S. esocinus* followed by *S. labiatus*. The variations in the prevalence of *A. oreini* and *P. kashmirensis* in different species of *Schizothorax* may be attributed to the sharing of common breeding habitat, feeding habit, dietary preference for intermediate host by fishes, intraspecific differences in the physiology and resistance of the host as also suggested by many workers for wide host range of *P. kashmirensis*

and *P. bulbocolli* (**Amin 1987b; Chishti and Peerzada, 1998**). Many species of acanthocephalans are known to alter the behaviour of intermediate host in a manner that could increase their transmission to the definitive host (**Nickol, 1995**).

It was further observed that the prevalence rate of both parasites under study varies with the season. The prevalence of *A. oreini* was found more or less same during spring, autumn and winter, whereas, exceptionally high during summer which may be due to variation of temperature in water bodies, availability of infected intermediate host, amount of food consumed by the fishes, as they feed most actively during summer (**Dogiel, 1958**). The higher prevalence of *A. oreini*, particularly during summer may also be determined by the longevity of tapeworm larvae in the intermediate host, time of predation of the fish host and rapid development of the parasite within host during this season as suggested by **Hanzelova and Gerdeaux (2003)** for *P. longicollis*. Contrary to this, **Khan *et al.*, (2004)** reported higher prevalence of *A. oreini* during spring. **Dhar and Peerzada (1992)** reported that recruitment of *A. oreini* infection in fish started in September and infra population of this parasite increased in winter. These workers also suggested that the seasonal incidence cycle was due to temperature variations. Seasonal variation in the prevalence of other caryophyllidean cestodes have also been reported by many workers which was correlated with the temperature variation and changes in the population and seasonal disappearance of intermediate hosts (**Kennedy,**

1968; 1969a, b; Smith, 1973; Henricson and Nyman, 1976; Esch, 1983; Marcogliese and Esch, 1989).

Belghyti *et al.*, (1994) suggested that the origin of seasonal variations of helminth parasites in fishes may be due to host trophic variations and population of fish host, and the abundance of parasite increases before the active reproduction period when food intake increases. **Kennedy and Walker (1969)** have shown experimentally that the establishment and survival of *C. laticeps* in Dace is better at low than high temperature and suggested that the resistance of fishes may be affected by temperature changes, making the fishes more susceptible during the winter months. Further, **Granath and Esch (1983)** reported seasonal variation in the occurrence of ***B. acheilognathi*** in different fish species, as this parasite was found during winter and absent during summer. On this basis, they suggested that the temperature of water bodies is the main factor for the establishment of parasite and not the availability of intermediate hosts as, they are available throughout the year. Such phenomena are not operative in the case of ***A. oreini*** and infection of this parasite in the fishes was observed round the year. Therefore, the feeding habit and fluctuations in the population of intermediate host may be the main factor for seasonal variations in the prevalence of this parasite as also suggested by **Belghyti *et al.* (1994)** for *B. anderseni* in *C. lingulata*.

A seasonal cycle in the occurrence of ***P. kashmirensis*** was also observed. The prevalence of ***P. kashmirensis*** was found higher during autumn,

slightly lower during winter and spring and very low during summer (June, July, August), which indicate that seasonal changes have more influence on the prevalence of this parasite as compared to *A. oreini*. Comparatively low prevalence of ***P. kashmirensis*** during summer may be due to higher water temperature which possibly cause an increase in parasite mortality and limit the successful establishment of parasites in their definitive hosts as, observed in other species of acanthocephalans (**Awachie, 1966; Kennedy, 1972a; Belghyti *et al.*, 1994**). Similarly, many workers also reported the seasonal cycle in *P. kashmirensis* and high prevalence was recorded during spring (**Chishti and Peerzada, 1998; Khan *et al.*, 2004**). A seasonal nature of parasitism was also described in other fish acanthocephalans (**Amin, 1975; 1987b; Camp and Huizinga, 1980; Chubb, 1982; Nickol, 1995**). Low prevalence and intensity of infection of *P. bulbocolli* has been reported during autumn and higher during summer (**Lawrence, 1970; Boxrucker, 1979**) which was correlated with the extreme temperature fluctuations during different seasons. Seasonal periodicity in abundance of *N. cristatus, P. bulbocolli* and *Octospinifer macilentus* has also been reported in *C. commersoni* from one of two sites in New Hampshire (**Muzzall, 1980a**), which was attributed to the movement of larger spawning fish. **Belghyti *et al.*, (1994)** reported more than 50% infection of *A. propinguus* in *Dicologoglossa cuneata* throughout 16 months which was correlated with the number of amphipods included in the diet of fishes, and not affected by season or age of fish. High prevalence of *P. bulbocolli* was

recorded in *C. carpio*, *Ictalurus* spp. and *Moxostoma* spp. during summer and in *C. commersoni* during autumn **(Amin, 1987b)**. He further reported that the infection patterns were influenced by fish species, feeding behaviour, temperature, availability of intermediate host, type of water body, fish movement and changes in fish host community. The high prevalence of *P. bulbocolli* during summer was correlated with the feeding activity of fishes which increases in warmer temperature and thereby causing more ingestion of crustacean host. Such suggestion is true for *A. oreini* but not for *P. kashmirensis*, as the infection of *A. oreini* was found higher during summer and *P kashmirensis* during autumn. Thus, many other factors may be responsible for the seasonal variations of *P. kashmirensis* infection.

The seasonal variations in the prevalence of *P. kashmirensis* may also depend on the seasonal changes in the density of intermediate host containing cystacanth and number of intermediate host consumed by fishes as suggested by **Camp and Huizinga, (1980)** for *A. dirus* infection in Creek Chub. It was also shown that the amphipods (*G. pulex*) infected with *P. laevis* are eaten by fish in significantly greater proportion than uninfected individuals, even when infected amphipods constitute a very small fraction of the amphipod populations **(Kennedy *et al.*, 1978)**. It has been shown that the transmission of *P. longicollis* larvae from copepod to common white fish (*Coregonus lavaretus*) took place most intensely in summer and autumn and depended on seasonal changes in the density of the *Cyclops abyssorum prealpinus*

population, infection of this copepod with plerocercoids and their density in the lake **(Hanzelova and Gerdeaux, 2003)**. The eggs once released by the parasites, take many weeks to infect the intermediate host because the infective stage develops after several months **(Kulakowskaja, 1962)**. Though, the infection is present in intermediate host, but they are not infective till the development of infective stage. The development of larval stages in the intermediate host are temperature dependent and it may take more than a year depending upon temperature of water bodies **(Awachie, 1966; Tokeson and Holmes, 1982; Brattey, 1986)** and therefore, cause variation in the infection to definitive host.

Thus, it can be concluded from the above discussion that the extreme trends in infection of different species of acanthocephalan in different habitats and fish species may be due to versatility in feeding of fishes and variations in the invertebrate community and availability.

When the present data were analyzed on the basis of occurrence of both parasites in the same individual, more females were found infected with high worm burden than males. Such differences are probably due to females eating more than males as a result of physiological and behavioral differences, as also suggested by **Thomas (1965)** for the trematode, *Mesocoelium monodi* infection in lizard. He also suggested that females were less likely to maintain a high body temperature which might be detrimental to the parasite.

When the occurrence of individual parasite was analyzed in individual

fish host, it was observed that sex of the host has insignificant influence on the incidence of *A. oreini* and *P. kashmirensis*, as the prevalence of these parasites was slightly higher in male than female fishes. Similar results were also reported for other caryophyllidean cestodes **(Lawrence, 1970; Muzzall, 1980b; Belghyti *et al.*, 1994)** and acanthocephalans **(Amin, 1975; Muzzall, 1980a)** infecting different species of fishes at different sites. Contrary to the present findings, higher prevalence of *P. bulbocolli* **(Lawrence, 1970; Amin, 1987b)**, *N. prolixoides* **(Amin, 1986)** and *A. propinquus* **(Belghyti *et al.*, 1994)** has been reported in female than male fish.

The prevalence and intensity of both parasites increase with the increasing size of fishes. Usually larger fishes (20-30cm) were found more heavily infected than smaller (< 20cm) and very large (>35cm) fishes. Similar results have also been reported by many workers with caryophyllidean and acanthocephalan parasites **(Muzzall and Bullock, 1978; Muzzall, 1980a; b)**. Contrary to this many workers either found decreasing trend or insignificant relationship between prevalence of many acanthocephalan parasites and size or age of the fishes **(Lawrence, 1970; Amin, 1987b, Belghyti *et al.*, 1994)**. The increase in the prevalence rate with size of fish may be due to an increase in the number of infected intermediate host eaten by larger fish, increased food volume with increasing size, change in feeding habit with change of host length as also suggested for *G. catastomi* and *P. bulbocolli* **(Muzzall, 1980a, b)**. As the length of fishes increase, the size of intestine also increases, therefore there

is more space and surface area to attach to and occupy. Since, larger fish are feeding more therefore, the probability of their eating infected intermediate host is increased, causing an increase in prevalence rate. Similarly, increased food volume containing the intermediate host has been reported in the fishes of 20 to 25 cm length **(Amin, 1975)**. Similar suggestions have also been made by many workers for the abundance of parasites in different length groups of fishes **(Hine and Kennedy, 1974; Des Clers, 1991; Belghyti *et al.*, 1993; 1994; Chishti and Peerzada, 1998)**. Similarly, **Amin (1986)** reported maximum infection of *N. prolixoides* among the fishes having 23 to 28 cm length and correlated it with increased consumption of food including infected intermediate host. A similar close relationship between diet and parasitism was also established in cestode *P. filicolius* **(Hopkins, 1959)**, *B. ranis* **(Jaroll, 1979)** and *B. greganius* **(Robert *et al.*, 1990)**. In very large fish (over 30cm long) prevalence and intensity decline which may be due to age immunity **(Wakelin, 1985)** or due to changes in diet composition of fishes of different age as suggested by **Amin (1974)** in *C. lingulata*. **Belghyti *et al.*, (1994)** reported that prevalence and intensity decline in the fishes over 150 mm long and correlated it with the acquisition of sexual maturity and to increasing piscivory. A progressive decrease in parasitism may also be linked to an unbalanced parasite recruitment and death as observed in *Osterlogia osterlogi* **(Michel, 1970)**.

The mean intensity of both parasites was found to increase up to 30 cm

length and then decreases. Similar result was also reported by **Muzzall (1980a)** for *O. macilentus* in *C. commersoni*, where mean intensity was found to increase up to 34 cm and then decreases. Two high peaks of mean intensity were observed for *A. oreini* which was similar to those reported by **Muzzall (1980b)** for *I. bulbocirrus*. The high mean intensity particularly of *P. kashmirensis* in 20 to 30 cm length fish may be due to the overlapping of female generations, because females live longer than males **(Parshard and Crompton, 1981)**. Thus, overlapping may occur between initial generation of females and the newly recruited generation of males and females. The progressive decrease of the intensity could then be due to death of females in the first generation. Similar suggestion has also been made by **Belghyti *et al.*, (1994)** for the variation in intensities of other acanthocephalan parasites with season. Low intensity may be explained by the fact that the intermediate hosts of these parasites carry very few infective stages as suggested for other helminth parasites of fishes (See references in **Belghyti *et al.*, 1994**).

The distribution of both the parasites in their definitive hosts was over-dispersed. It can be concluded from the observed data that the parasites were not dispersed at random, because small number of animals were found to harbour most of the parasites. The aggregated distribution of parasite in the host is a common feature of host-helminth system and depends on a number of host and environmental factors which act together to cause heterogeneity in infection **(Munro *et al.*, 1989)**. Over-dispersion of hydatidosis has been

demonstrated in buffaloes from India (**Irshadullah *et al.*, 1989**) and in cattle, sheep and goat from Kenya (**Macpherson, 1985**) where, it was suggested that the condition was due to the special aggregation of *Echinococcus* eggs, the infectivity of such eggs and differences in the susceptibility of various hosts to infection. Recently, **Tingbao and Xianghua (2001)** suggested that over-dispersed distribution of *N. qinghaiensis* in *Gymnocypris przewalskii przewalskii* in Qinghai Lake, China, was due to heterogeneity and feeding habits of the host. The main factors governing over-dispersion are thought to be the efficacy of the individual's immune response against invading parasites **(Wakelin, 1985)**, the spatial distribution of infective larval stages in the environment, individual host behaviour patterns **(Keymer and Anderson, 1979)**, heterogeneity between hosts, their exposure and susceptibility to infection or defensive capabilities **(Anderson and Gordon, 1982)**.

Since, the infection in smaller fishes (<5.0 cm), occurrence of juvenile stages of parasites in fishes and intermediate host, population dynamics of intermediate hosts and their role in the transmission of disease were not included in the present study. Therefore, further studies are required on these lines in order to suggest an appropriate epidemiological based control model for these parasites.

[II] Histopathological Studies:

It is evident from the results that *A. oreini* are not firmly attached to the intestine of the host as they do not have any special attachment organ and remain in the lumen of the intestine. It may be possible that they migrate up and down and between the villi like other cestodes **(Rees, 1967; Featherstone, 1969; Coman and Rickard, 1975; Beveridge and Rickard, 1975; Dick and Choudhury, 1995)**. The pathology in the gut is often related to the morphology of the holdfast organs of the parasite. Those species of Caryophyllidea that possess well developed but non-invasive attachment organs elicit little or no pathology whereas, those having terminal introvert on the holdfast produce ulcers and nodules **(Mackiewicz *et al.*, 1972)**. The caryophyllidean cestodes produce heavy losses to the fishes by causing inflammation, mechanical obstruction, perforation, thinning of intestinal wall and sometimes death due to dysfunctioning of intestinal mucosa. In heavy infection, the diameter of the lumen of intestine is reduced by more than 50% which affects the movement of the food and thereby causing death of the host **(Scott and Grizzle, 1979; Bauer *et al.*, 1981; Shostak and Dick, 1986)**. In the present study, atrophy of villi and flattening of mucosa in the infected fish intestine was observed which might reduce surface area for absorption. Similarly, the loss of surface area in the rainbow trout, rat and sheep intestine due to villus atrophy has also been reported in many parasitic infections **(Barker, 1973; Symons, 1976; Williams and Jones, 1994)**. Changes in the absorptive surface area may be responsible

for impaired digestion and absorption of food, water and electrolytes. A similar suggestion was also made for intestinal trichinosis and trichuriasis (**Castro *et al.*, 1967; Mathan and Baker, 1970**) and the degree of malabsorption depends on the severity of infection (**Symons *et al.*, 1971**). The impaired intestinal absorption of food, electrolytes and water may also be a consequence of reduction in the "pore size" of mucosal membrane in many parasitic infections (**Mettrick and Podesta, 1974**).

In the present study some specialized gland cells were observed in the scolex region of *A. oreini* which probably release secretary materials at the host parasite interface and help in establishment of the parasite. Similarly, it has been suggested that *Adenoscolex* attached with the gut epithelium by the adhesive secretion from gland cells of scolex which are extended up to three quarter of anterior body length (**Fotedar, 1958; Fayaz and Chishti, 1998**). **Hayunga and Mackiewicz (1988)** reported that the scolex gland was more developed in those species which lack special attachment organ and suggested that the secretion of the glands was used by the parasite to adhere with host intestine. Such secretory cells have also been reported in other cestodes (**Farooqi, 1958, 1986; Smyth, 1964a; Andersen, 1975a, b; Hayunga, 1979; Thompson *et al.*, 1979; Specian and Lumsden, 1981**). At present it is difficult to assign any specific function to such gland cells however, on the basis of their location and cytoplasmic nature, different speculations can be made. The secretion may cause lysis of host tissue and distortion of the villi for

deeper penetration of scolex and may act as functional antigen, as suggested for *E. granulosus* (Smyth, 1964b; Rickard, 1983; Kanwar and Vinayak, 1993). Therefore, it is proposed that the immunological role of the secretion should be investigated. Besides this, different authors suggested that the secretion of scolex glands are involved in various physiological functions such as nutrition, adhesion, maturation of ova, protection of the parasites against host's digestive enzymes and mitochondrial biogenesis in other cestode species (Farooqi, 1958; 1986; Smyth, 1964a ; Jha and Smyth, 1971; Öhman-James, 1973; Andersen, 1975a; Thompson *et al.*, 1979; Gustafsson and Vaihela, 1981).

In contrast to *A. oreini*, more complex host's reactions were observed due to *Pomphorhynchus* infection, particularly around the proboscis and bulbous neck. *P. kashmirensis* have well developed hooked proboscis with which they firmly attach to the intestine of the host. This species penetrates its armed proboscis and bulbous neck deep into the mucosal epithelium and between the villi and thereby causing more damage to the villi and lamina propria, as also reported for other acanthocephalan parasites of fishes (Chaicharn and Bullock, 1967; McDonough and Gleason, 1981). Polzer and Taraschewski (1994) reported that the cystacanths and adult *P. laevis* release proteolytic enzyme and suggested that this enzyme may be involved in the degradation of the host tissue. Collagen was the major molecule degraded by the enzyme of *P. laevis* cystacanth. Thus, on the basis of damages observed in the present study, it can be suggested that *P. kashmirensis* also release

proteolytic enzyme like *P. laevis* and consequently cause more damage to the tissues of gastrointestinal tract as compared to *A. oreini*. However, further studies are required to confirm this hypothesis. Destruction of villi and degenerative changes in mucosal epithelium adversely affect the absorptive efficiency of the fish intestine which may affect the general health and growth of fishes. At the site of attachment, host cells are destroyed and many cells like fibroblasts, lymphocytes and macrophages are mobilized below the lamina propria **(Dezfuli, *et al.*, 1990)**. Furthermore, chronic fibrous inflammation leading to an increased amount of connective tissue and thickening of lamina propria have been reported at the site of attachment of *A. jacksoni* **(Bullock, 1963)**. In some species, fibroplasia extends to the layers of the muscularis mucosa **(de Buron and Nickol, 1994)**. **Bullock (1963)** compared the pathogenecity of *A. jacksoni* in different species of fishes and reported that the parasite causes more damage in *S. gairdneri* which is not a suitable host for this parasite. Thus, the degree of pathogenecity and intensity of host reactions depends on the parasite species and site of localization **(Esch and Huffines, 1973; Hine and Kennedy, 1974; Hamers *et al.*, 1992)**. The presence of bulbous neck in *P. kashmirensis* prevents its subsequent intestinal migration as also reported by **Dezfuli (1991)** for *P. laevis*.

The cellular infiltration around the vicinity of worms indicates the involvement of cell mediated immunity against the parasite. Such accumulation of host cells have also been reported by many workers due to various parasitic

infections (**Smyth *et al.*, 1969; Hoole and Arme, 1983; Sharp *et al.*, 1992**). The infiltrated cells were differentiated as lymphocytes, neutrophils and plasma cells, as also demonstrated in different cestode infection (**Lumsden and Karin, 1970; Pappas, 1976; Taylor and Hoole, 1989; Sharp *et al.*, 1992**). In addition to this, the number of goblet cells increase due to *A. oreini* infection as reported in *Ligula* and *Schistocephalus* infections (**Andersen *et al.*, 1987; Andersen and Gibson, 1989; Grabda, 1989**). Similarly, it has been shown in green sunfish (*Lepomis cyanellus*) infected with *Leptorhynchoides thecatus* that the number of goblet cells was significantly higher in parasitized pyloric caeca than unparasitized caeca in the same fish (**de Buron and Nickol, 1994**). These cells secrete excessive mucus in infected intestine which is a common phenomenon in parasitic infection and could provide defense to the host (**Nickol, 1995**).

Intense host response, like the formation of fibrous capsule and infiltration of granulocytes and fibroblasts was noticed at the site of attachment of *P. kashmirensis*. Such response has also been reported due to the infection of other species of *Pomphorhynchus* in fishes (**Esch and Huffines, 1973; Hine and Kennedy, 1974; McDonough and Gleason, 1981; Chishti *et al.*, 2002**). **Hamers *et al.*, (1992)** found interspecific differences in the response of leucocytes in fishes parasitized with *P. ambiguus* and suggested that the cellular defense may be involved in determining the host specificity as unsuitable host expel the parasites within a few days. Some species of

Pomphorhynchus form fibrotic tunnel instead of capsule in the intestine of the host. In those areas where the trunk of the parasite was in contact with the host tissue, the epithelial cells were found compressed and eroded. Similar changes have also been reported in the intestine of a number of fishes infected with acanthocephalan parasites **(Bullock, 1963; Chaicharn and Bullock, 1967; Nickol, 1995)**. Due to such damage the surface area for absorption decreases by which many physiological disorders appear in the host as discussed above for *A. oreini*.

[III] Pathophysiological Studies:

The results of pathophysiology reveal that many blood components of non-infected fish sera vary with the season which may be due to the effect of various abiotic factors. In order to find out the effect of parasitism on the level of various blood components, percent change was calculated with their respective controls during different season. The variations in percent change suggest that any increase or decrease in the blood components was due to the effect of parasitic infection and abiotic factors, as effect of the latter is common in both infected and non-infected fishes inhabiting same habitat. The minor fluctuations in blood components with respect to seasons can be correlated with the physiological and metabolic adaptation of fishes under different climatic conditions, sexual maturation and breeding cycle. Similarly, the fluctuation of blood chemistry during different season was correlated with seasonal changes

in salinity, dissolved oxygen, water temperature, food availability and sexual maturation (**Miller *et al.*, 1983; Sandnes *et al.*, 1988; Folmar *et al.*, 1992**). It has also been reported that handling stress (**Wedemeyer and McLeay, 1981**), choice of anesthesia (**Houston *et al.*, 1971; Laidley and Leatherland, 1988**) and method of blood withdrawal (**Gaudet *et al.*, 1975**) may also alter some blood chemistry parameters.

(i) Serum enzymes: The fluctuations in the level of sGOT, sGPT, serum acid and alkaline phosphatases in non-infected fishes during different seasons can be correlated with the fluctuations in water temperature. Similarly many workers reported that the level of these serum enzymes fluctuate with the variation of temperature and suggested that such changes were due to cold stress and inactivation of enzymes at higher temperature (**Sauer and Haider, 1977; Sandnes *et al.*, 1988; Kapila *et al.*, 2002**). The higher level of acid phosphatase at 20 and 30°C was due to leakage of this enzyme from RBC due to increase in its membrane fragility by thermal stress (**Kapila *et al.*, 2002**). Further, the activity of these serum enzymes was shown to be influenced by species of the fishes (**Goel *et al.*, 1981; Casillas *et al.*, 1982**).

The significant fluctuations of sGOT, sGPT, serum acid and alkaline phosphatase in infected fishes with respect to seasons can be correlated with the recruitment and establishment of infection, as significant changes in the level of these enzymes were reported in early (establishment period) and late phase (egg production) of *E. granulosus* infection in experimental puppies

(Irshadullah, 1994). The summer and spring peaks of sGOT and sGPT particularly in ***P. kashmirensis*** infection may be due to establishment and maturation of worms, as during summer only adult ***P. kashmirensis*** was recorded. Thus, it can be concluded that the spring and summer would be the time of recruitment of infection and worm maturation, respectively. However, further studies are required to assess the exact time at which establishment and maturation occur. It is difficult to explain minor fluctuation of these enzymes during different seasons, however, it can be speculated that these changes are due to adaptation of fishes under different environmental conditions as the metabolic process changes with the variation of temperature **(Joshi *et al.*, 1980; Smith *et al.*, 1981)**.

The percent change of various enzymes in the fishes infected with ***A. oreini*** and ***P. kashmirensis*** with their respective control clearly indicate that the variation was due to the presence of parasitic infection. Such changes have also been reported in other parasitic infections in fishes as well as other vertebrates **(Sapozhnikov, 1969; LeBars and Benting, 1976; Kurovskaya, 1984; Holmes, 1986; Williams and Jones, 1994)**. It was shown that both ***B. acheilognathi*** and ***K. sinensis*** disrupt intestinal and liver enzyme activity in ***C. carpio*** and the morbidity and mortality were attributed to the changes in alanine and aspartate aminotransferase activity which interfered with protein synthesis **(Lozinska-Gabska, 1981)**. The percent increase in the level of various serum enzymes due to these parasites can be correlated with the

mucosal damage caused by the parasite during the process of their establishment as acute inflammation, decreased vascularisation, tissue alteration/destruction and epithelial hyperplasia have been reported due to intestinal tapeworms (reviewed by **Dick and Choudhury, 1995**). It may be possible that, due to such damage there is leakage of various enzymes from infected tissues into serum. The mucosal damage induces decreased level of brush border enzymes in parasitized intestine (**Jones, 1983**). Similarly, increase of certain serum enzymes was correlated with the formation of lesions by *T. nodulosus* infection in fishes (**Scheinert and Hoffmann, 1986**).

Alkaline phosphatase, GOT and GPT belong to the non-plasma specific enzymes which are located within tissue cells and have no known physiological functions in plasma (**Tietz, 1976**). When cell membranes are intact, these enzymes are present in serum in low concentration, as the cell membranes are impermeable to enzymes. Following cell damage, the membranes become permeable and enzyme activity is found in extra-cellular fluid and serum (**Sandnes *et al.*, 1988**).

During the course of present study, it was observed that the level of sGPT decreases while acid and alkaline phosphatases increase in fish sera infected with *A. oreini* as compared to controls, whereas, in *P. kashmirensis* infected fish sera, a reverse phenomenon was found. Thus, on this basis, it can be suggested that these enzymes can be used as marker to detect the infection of these parasites under field conditions as, the determination of sGOT, sGPT

and alkaline phosphatase was found to be useful in the diagnosis of liver, bones and kidney diseases in fish (**Racicot *et al.*, 1975; Teitz, 1976; Maita *et al.*, 1984**). Maximum increase in the level of the sGOT and sGPT in ***P. kashmirensis*** than ***A. oreini*** infection may be due to liver damage, as it was reported that ***P. kashmirensis*** and other species of ***Pomphorhynchus*** perforates the intestine and penetrates its proboscis in the liver (**McDonough and Gleason, 1981; Dezfuli, 1991; Chishti *et al.*, 2002**).

(ii) Serum Proteins: The level of total serum proteins, albumin and globulin showed considerable variation in infected animals with respect to control. The levels of total serum proteins and albumin in infected fish sera significantly decrease and globulin increases with respect to control. Similarly, many workers reported the loss of total serum proteins and amino acids in *C. carpio* and *C. batrichus* infected with other caryophyllidean cestodes (**Sapozhnikov, 1969; Kudryashova, 1970; Kadav and Agarwal, 1982; Kurovskaya, 1984**). **Soutter *et al.*, (1980)** reported significantly lowered concentration of a number of blood amino acids as a consequence of parasitism and suggested that the effect of ***Ligula*** on host amino acids were comparable to those occur in starvation. The changes in total serum proteins may be due to the changes of various protein fractions and its source as it was reported that serum proteins have different sources (**Sandnes *et al.*, 1988**). The decrease in serum albumin and increase in β and gamma-globulin has been reported in ***Ligula*** infected *A. brama* (**Guttowa and Honowskawa, 1973**), *T. bovis* infected calves (**Cena,**

1976) and *T. pisciformis* infected rabbits (**Liebermann and Boch, 1960; Chevrier *et al.*, 1971**), which was associated with the damage of liver and migration of many species of parasites and was non-specific.

The decreased level of albumin can be correlated with the leakage from damaged epithelial mucosa due to increased permeability of capillaries as it is a common phenomenon in many parasitic infections (**Nielson, 1976, Von Brand, 1979**). Gastro-intestinal leakage of plasma proteins particularly albumin is a well established fact in a number of parasitic infections in the small intestine (**Symons, 1982**). Similarly, deficiencies in blood proteins were found in hookworm and other parasites that feed on blood and especially those that cause enteric haemorrhage (**Hall, 1985; Holmes, 1987; Symons, 1989; Holmes and Zohar, 1990**). Since, serum proteins have different sources, but albumin is synthesized only in the liver therefore, the decreased level of albumin in infected than non-infected fish may be attributed to the decreased synthesis in the liver due to effect of parasitism. The higher levels of globulin indicate the involvement of humoral immunity against this parasite. Many workers have also recorded increase in serum globulin from infected fishes (see references in **Buchmann *et al.*, 2001**). The serum proteins have a variety of functions and are especially important for regulation of water balance in fishes (**Wedemeyer and Yasutak, 1977**), therefore, the above discussed changes in serum proteins due to parasitic infection may prove to be deleterious to the fishes.

(iii) Serum Lipids: The serum lipid profile was also found to be affected by seasons and parasitic infection. The variations in the level of total serum lipids, triglycerides, cholesterol, HDL and LDL in non-infected animals can be correlated with the feed intake, diet and reproductive cycle of the host, as suggested by **Deb *et al.*, (1983); White *et al.*, (1983); White and Fletcher, (1985); Sandnes *et al.*, (1988)**. Similarly, many workers reported initial decrease in the level of serum cholesterol during spawning **(McCartney, 1966; 1967; Tandon and Chandra, 1976; Joshi, 1980; Singh and Singh, 1984)** and total serum lipids during vitellogenesis, followed by a rapid rise on the onset of spawning and then a rapid decline in many fish species **(Petersen and Emmersen, 1977; Sand *et al.*, 1980; Sheridan *et al.*, 1983)**. **Dannevig and Norum (1982)** did not find any definite pattern of variation in the plasma cholesterol of ***S. alpinus*** but did find an increase in the percentage of esterfied cholesterol during the prespawning and spawning period. Since, cholesterol is a precursor for the synthesis of sex steroids, therefore, the levels may change during the reproductive cycle **(Felinska 1972)**. A high level of low and high-density lipoproteins in ***Onchorhynchus*** was reported before spawning **(Nelson and Shore, 1974; Eaton *et al.*, 1984)**.

It was further observed that the level of total serum lipids, triglycerides, cholesterol and LDL were significantly higher in infected than their respective control. Similar results have also been reported in parasitized hamsters and rats with ***S. mansonoides*** larvae **(Phares and Carroll, 1977; 1978)**. Increased level

of serum lipids may be due to the effect of parasitism on the lipid metabolism of the host, as suggested by many workers for other cestode parasites (see references in **Arme *et al.*, 1983**). It has been reported that the plerocercoids of ***S. mansonoides*** produce a growth promoting substance (probably proteins of approximately 70KD molecular weight) which enhances the growth of many parasitized animals as well as affect the lipid metabolism of host (**Arme, 1975; Arme *et al.*, 1983**). **Ruegamer and Phares, (1974)** reported increased concentration of total serum lipids and triglycerides in parasitized female rats with the larvae of ***S. mansonoides*** and castrated males, and on the basis of similar composition of fatty acids in the host and parasite they suggested that the plerocercoid might alter host lipid biochemical pathways to provide appropriate substrates for its own metabolism.

Since, the liver plays a key role in the metabolism of triglycerides and cholesterol (**Sandnes *et al.*, 1988**) therefore, any changes occurring in the liver may cause similar changes in the level of these components.

[v] Glycogen and Proteins of infected fish muscle and liver: The analysis of protein and glycogen in infected fish liver and muscle was found significantly less as compared to non-infected fishes which may be due to deficiency of monomers used for glycogenesis and protein synthesis. Recently, **Soliman *et al.*, (2004)** reported significant decrease in the level of glycogen, protein and lipid in infected fish muscle and liver with heterophyid metacercaria and undifferentiated nematode larvae. They further reported fluctuations in the

banding patterns of proteins by SDS-PAGE and suggested that the fluctuation in protein bands might be a reflection of stress or impact on ribosome and RNA levels and consequently on protein synthesis. **Walkey and Meakins (1970)** found that much of the energy taken in by the infected fish is redirected to the production of parasite tissue. This energy loss results in the depletion of the endogenous reserves of the host and a higher metabolic demand on the infected fish **(Lester, 1971; Meakins, 1974; Meakins and Walkey, 1975)**. **Connors and Nickol, (1991)** suggested that acanthocephalan *Plagiorhynchus cylinderceus*, causes significant detrimental effect on the flow of food energy through the host and alters its basal metabolism.

Maximum decrease in the level of glycogen and protein in the fish muscle infected with *A. oreini* and *P. kashmirensis* respectively, can be correlated with the mobilization of these components for energy production as suggested by **Wanstall *et al.*, (1982)** for *P. laevis* infection in *S. gairdneri* and *N. barbatulus*. These workers reported that muscle and liver proteins are significantly influenced by parasite burden whereas, lipid and carbohydrate levels remain unaffected and there was a threshold level of infection below which protein depletion does not occur. Further, the depletion of host protein by *P. laevis* was attributed with the host spawning, reduced winter feeding, competition between host and parasite for food, increased gluconeogenesis from muscle protein due to the production of corticosteroids in response to infection and host's tissue response to the parasite. The present findings can be

correlated with the competition between host and parasite for food and host's tissue response to the parasite but not with other factors as, both proteins and glycogen were found to decrease significantly

[IV] Haematological Studies:

Besides the biochemical changes in the serum, muscle and liver of infected fishes, some haematological parameters were also investigated. The loss of RBC can be correlated with the penetration of proboscis and haemorrhagic condition, as intestinal haemorrhage has been reported in many helminth infections **(Rees, 1967)**. Similarly, massive loss of RBC due to haemorrhage has been reported in various helminth infections **(Holmes, 1987)**. The other possible reason for decreased level of RBC counts could be due to the production of toxins, which influence the life span and production of RBC **(Horak *et al.*, 1968)**. It may also be possible that vitamin B_{12}, an essential component for erythropoiesis is either not absorbed by the parasitized intestine due to impairment of intrinsic factor secretion or utilized by the parasite **(Von Bonsdorff, 1956; Nyberg, 1963; Gardiner, 1966; Salokannel, 1970)**. Large worms located in the intestine can absorb vitamin B_{12} secreted in the stomach prior to its reabsorption in the ileum, thus inducing a condition similar to pernicious anaemia **(Hall, 1985; Holmes and Zohar, 1990)**. Vitamin B_{12} is apparently taken up by almost all tape worms except cyclophyllideans and this ability was correlated with the use of B_{12} as a co-factor in CO_2 fixation for

energy metabolism (**Arme *et al.*, 1983**). Similarly, iron deficiency anaemia may be produced by hookworms or other parasites that feed on blood, especially those that cause enteric haemorrhages (**Holmes and Zohar, 1990**). **Sinclair (1965)** suggested that anaemia is caused by the disturbed activity of reticulo-endothelial system which may lead to the decreased production and increased destruction of erythrocytes.

Increase in total leucocyte count suggests the involvement of cell mediated immune response as it was suggested that the lymphocytes release a chemotactic factor which helps in the accumulation of eosinophils and neutrophils and may cause damage to the parasite (**Dineen and Kelly, 1973; Butterworth, 1984; Anuradha and Katiyar, 1987**). It was observed that the number of neutrophils and lymphocytes increases due to infection of both parasites whereas, monocytes decrease in *P. kashmirensis* infected fishes which may be due to metabolic and antigenic activity of the worm (**Blazek *et al.*, 1981**). Increase in monocytes and polymorphonuclear leucocytes was reported in *Ligula* infection in fish (**Shpolyanskaya, 1953**). Elevated leucocyte and phagocyte counts and appearance of giant lymphocytes were reported in *C. carpio* infected with *B. acheilognathi* (**Kudryashova, 1970; Kirchenko and Kosareva, 1972; Par, 1978; Svobodova, 1978**). Many workers reported decrease in erythrocytes, monocytes and neutrophils due to *K. sinensis* and *E. salvelini* in *C. carpio* (**Hoffman *et al.*, 1986**) and there was a significant correlation between the degree of haemosiderosis in the spleen and the

intensity of infection. Further, the decrease in haemoglobin content due to both parasites under study is similar to those reported in other caryophyllidean cestode infections in fishes (**Sapozhnikov, 1969; Kadav and Agarwal, 1983; Williams and Jones, 1994**). The fall in the haemoglobin may be due to loss of RBC. Many workers also reported fall in haemoglobin concentration and PCV due to many helminth infections in vertebrate host (**Kadhim, 1976; Arme *et al.*, 1983**). **Härdig and Høglund, (1983)** suggested that haemoglobin synthesis occurs in the erythrocytes after their release into the circulation. It has been shown that many factors like nutritional status (**Barnhart, 1969; Spannhof *et al.*, 1979**), infectious diseases (**Amend and Smith, 1975; Barham *et al.*, 1980; Iwama *et al.*, 1986**), environment (**Goel *et al.*, 1981; Munkittrick and Leatherland, 1983; Giles *et al.*, 1984; Iwama *et al.*, 1986**) and stress (**Yamamoto *et al.*, 1980; Lowe-Jinde and Nimii, 1983; Wells *et al.*, 1984; Ellsaesser and Clem, 1986**) have considerable influence on the fish haematology and therefore these factors must be taken into account in further studies.

The foregoing discussion reveals that some haematological changes occur in the host due to parasitic infections. These changes are non-specific and cannot be used as diagnostic markers for *A. oreini* and *P. kashmirensis*. It has been reported that many haematological parameters change with the variations in age (**Conroy, 1972**), season (**Härdig and Høglund, 1983**), strain (**Barnhart, 1969**), sexual maturation (**Lane, 1979**) and parasitic infection

(**Arme *et al.*, 1983**). Thus, such variations must be considered while using the haematological parameters an aid for health control of fishes.

[V] Biochemical Composition and Protein Polymorphism of *Adenoscolex* and *Pomphorhynchus*:

The biochemical analyses reveal that all biochemical components were significantly higher in *P. kashmirensis* than *A. oreini* due to the variations in the metabolic state of the parasite as was suggested by **Abidi *et al.*, (1989)**. It has also been reported that the biochemical composition of the cestode parasites depends on the strain, host species, age of the cestode and its degree of maturation **(Smyth and McManus, 1989)**. The present results can not be compared with other studies as it is the first preliminary report on the biochemical composition of these parasites. However, many reports are available on other cestode and acanthocephalan parasites of fishes **(Von Brand, 1973; Sterry and McManus, 1982; Starling, 1985)**.

Among various biochemical components, glycogen is the major biochemical component of *A. oreini* and *P. kashmirensis* and the values obtained for these parasites are comparatively lower than the plerocercoid and adult *L. intestinalis* **(Sterry and McManus, 1982)**, *S. solidus* **(Hopkins, 1950)** and acanthocephalan *Neoechinorhynchus* **(Dunagan, 1964)**. Glycogen is major energy reserve in many cestodes and acanthocephalans **(Von Brand, 1979; Starling, 1985)**. Furthermore, it has been reported that the

glycogen level was significantly affected by the sex of the host (**Graff and Allen, 1963**) and sex of the parasites (**Körting and Fairbairn, 1972**). Further, it has been reported that the glycogen content of acanthocephalans change dramatically with host feeding behaviour (**Crompton, 1972**). Many workers have shown that the cestodes utilize carbohydrate as the major and possibly only energy substrate (see references in **Smyth and McManus, 1989**). The high levels of glycogen indicate the substantial energy requirement for maturation, egg production and maintenance of adult's position within the intestine of definitive host. **Bolla and Roberts (1971)** reported high carbohydrate concentration in the proliferative region of *H. diminuta* and suggested that somatic differentiation in the definitive host require higher amounts of carbohydrate. The high glycogen level in the parasites under study may also be due to low oxygen tension in the intestine of fishes as, it was suggested that those parasites which inhabit high oxygen tension possess low glycogen (**Barrett, 1981**).

The higher amount of lipids may be a biochemical adaptation to conserve energy requirements during emergency. **Sterry and McManus (1982)** reported abundant amount of proteins and lipids in the reproductive organs of *L. intestinalis* and suggested their role in egg production. **Vercelli-Retta *et al.*, (1975)** demonstrated increased level of lipids in the metabolic zones of cyst wall and suggested that the resultant products of lipid metabolism were important in the development and growth of the protoscoleces. A similar

suggestion could also be made for the level of lipids in these parasites. Different classes of lipids have been identified in many acanthocephalan parasites and it was reported that males have more lipids than females (reviewed by **Frayha and Smyth, 1983**). Further **Vykhrestyuk and Yarigina (1982)** suggested that variability in the lipid composition of parasites may depend on parasite species, sex, age and environmental factors such as habitat and diet of the host.

The lipid composition of cestodes and acanthocephalans possibly depends on the lipid composition of the hosts. It has been reported that sterols, sterol esters and fatty acids are absorbed by parasites from their host and then organized into lipid droplets by sub-tegumental cells (**Smyth and McManus, 1989**). **Byram and Fisher (1973)** observed inclusions of lipid droplets in the syncytial epidermis of *M. dubius*.

Lipids are the major components of cell membrane and play an important role in enzyme regulation, cell surface recognition, cell interaction, glycoprotein synthesis, expression of surface antigenic determinants and in membrane transport. Lipids often occur in association with carbohydrates and proteins as glycolipids and lipoproteins. Lipoproteins may be highly antigenic in cestodes as antigen 5 and antigen B, detected in the hydatid cyst fluid were lipoproteins (**Rickard and Lightowlers, 1986**).

The differences in the level of RNA and DNA between *A. oreini* and *P. kashmirensis* suggest variations in nature and metabolic state of the parasite,

as RNA constitute a marker for overall metabolic activity (**Smith and Walker, 1986**).

In addition to biochemical differences, both the parasites also differ in their protein profile analyzed by SDS-PAGE. Comparatively, more polypeptides were detected in *A. oreini* as compared to *P. kashmirensis*. Such differences could be due to differences in their genome. As it is a widely accepted fact that proteins are the first conceivable products of gene activity therefore, any variation in the polypeptides could reflect genetic constitution of the organism. Many workers were demonstrated substantial level of genetic variations between and within same strain by enzyme electrophoresis, restriction site analysis and DNA hybridization technique (**McManus and Smyth, 1979; McManus and Simpson, 1985; Lymbery and Thompson, 1988; McManus and Rishi, 1989; Irshadullah and Nizami, 1997**). Further, variations in number of polypeptides were also noticed in male and female *Pomphorhynchus*. The extra polypeptides detected in female worm could be associated with the process of egg production. Similarly, differences in protein profile has also been reported in male and female *Ascaridia galli* (**Saifullah *et al.*, 1993**).

It may be possible that enormous fecundity and niche segregation of helminthes might have led to the variation in protein profiles which ultimately reflect variation in their genetic constitution. **Kumaratilka and Thompson (1979)** also suggested that genetic constitution of parasite is reflected in their

characteristic protein profiles. Further, according to **Ferguson (1980)** the genetic composition of a population changes gradually over the generations due to mutation, over production of offspring and natural selection and only those genes are selected which are best adapted according to the condition of macro- and micro-habitats. It is also possible that some polypeptides could be of host origin as proteins of host origin have been detected at the surface of parasites **(Coltorti and Varela-díaz, 1974; Hustead and Williams, 1977; Hoole and Arme, 1986)** which resulted into antigen sharing and mimicry. In order to confirm this hypothesis, it is essential to identify the origin of proteins by radio-iodination technique.

The number of demonstrable polypeptides in any species probably depends upon the sensitivity of analytical technique, because the number of polypeptides varies with gel staining technique. Comparatively higher number of polypeptides were detected by silver staining which could be due to its higher sensitivity as well as staining property of conjugated proteins **(Porro *et al.*, 1982, Dzandu *et al.*, 1984)**. However, CBB R-250 dye is less sensitive for the detection of basic proteins as compared to neutral proteins and can detect calmodulin and troponin which can not be detected with silver stain **(Irie, *et al.*, 1982)**. Thus, different protein classes can be detected from the same gel by employing different staining procedures.

The specific polypeptides in male and female parasites of the same species not only represent the biochemical variations but also contribute to the

possible existence of antigenic variation. Thus the characteristic polypeptides can be exploited for immunodiagnostic as well as immunoprotective studies as **Simpson (1986)** pointed out that the heterogeneity in the polypeptide may be of considerable importance for the diagnostic and protective measures.

[VI] Immunological Studies:

Two and 4 precipitin bands were detected in the whole homogenates of *A. oreini* and *P. kashmirensis*, respectively by Ouchterlony's double diffusion technique. Further, the antibody titre was found quite high in the antisera raised against *P. kashmirensis* than *A. oreini* by ELISA. Such variations in the number of precipitin bands and antibody titre suggest that *P. kashmirensis* had comparatively more antigenic epitopes and high antigenic potential than *A. oreini* and thereby provoke more response. Cross reaction of antisera and antigens between *A. oreini* and *P. kashmirensis* did not occur which indicate that these parasites do not share any antigenic epitope.

The antibody titre also varied with the sex of the parasite, as the antibody titre of female *Pomphorhynchus* was higher as compared to male. Thus it can be suggested that the soluble homogenates of female parasite is more antigenic than male as suggested by **Adams *et al.*, (1988)** for *Nematospiroides dugius* and **Zeba (2002)** for *Setaria cervi*. Similarly, many workers demonstrated the production of specific antibodies by fish host against cestodes (**Sharp *et al.*, 1989**), monogeneans (**Buchmann, 1993, Wang *et al.*,**

1997, **Mazzanti *et al.*, 1999)** and acanthocephalans **(Harris, 1972)** where the antibody titre was correlated with the worm burdens.

When the sera of naturally infected fishes was tested against the antigens of these parasites, no reaction was observed. In contrast to this, **Chishti *et al.*, (2003)** have reported precipitating antibodies in the sera of naturally infected *S. niger* and *C. carpio* with these parasites. **Molnár and Berczi (1965)** using ouchterlony plates detected specific antibodies in infected *A. brama* with the plerocercoid of *L. intestinalis*, but **Sweeting (1977)** failed to detect precipitating antibodies in roach by using the same technique. Such variations could be correlated with the low antibody titre, sensitivity of the technique and seasonal variations. Many workers reported that production and level of antibodies in fish and other cold blooded animals against the antigens of helminthes and acanthocephalans was temperature dependant **(Kennedy and Walker, 1969; Harris, 1972; Alein and McDonial, 1973; Aaltonen *et al.*, 1997; Chishti *et al.*, 2003)**. In the present study, the naturally infected fish sera was not screened during different seasons, therefore, further studies are required to find out the effect of seasonal variations on antibody titre.

It may be possible that a large proportion of antibodies are adsorbed by the parasites to evade host immune response, as many workers suggested that immunoglobulins may be absorbed onto the surface of the parasite **(Varela-díaz and Coltarti, 1973; Coltarti and Varela-díaz, 1975)**. **Hoole and Arme (1983)** and **Fletcher *et al.*, (1980)** have suggested that the plerocercoid of

L. intestanilis and adult *B. scorpii* adsorb host proteins onto their surface to avoid the immune response of the host. It has also been reported that antibodies have no deleterious effect on the parasite because of adsorption of intact immunoglobulin by the parasite **(Threadgold and Hopkins, 1981)**. Hence, it is possible that a sufficient amount of antibody bound with the surface of the parasite and very little is available in the sera for immunological assay, which could not happen in the case of experimental (heterologous) host and therefore, antibodies were detected in hyper-immune sera.

Since, it is a preliminary study to find out the antigenicity of the two fish parasites by using hyper-immune sera raised in rabbits, further studies are required on natural host to develop immunodiagnostic tool as suggested by **Deplazes and Eckert (1996)** for *E. multilocularis*.

[VII] Topographical Effect of Drugs on *A. oreini* and *P. kashmirensis*:

After the treatment with drugs, apparent changes were noticed in the topography of scolex, proboscis, body surface and trunk of both the parasites. The drugs of different groups and concentrations were found to produce differential effects. Such differences can be correlated with the variation in the permeability of these drugs, binding with surface receptors and the detoxification of drugs by the parasites, by which they can reduce the toxicity of the drugs. Detoxification of anthelmintics have been reported in many helminth parasites **(Brophy and Barret, 1990)**. The differences in the nature

and degree of damage induced by various drugs may also be due to variation in their particle size (**Gordon, 1956**).

The benzimidazole derivatives, mebendazole did not produced any apparent topographical damage in *A. oreini* however, shrinkage, deeper grooves, lesions and peeling of tegument were noticed on ***P. kashmirensis***. The severity of damage increased with the concentration of drug. Previously, the effect of this drug was not tested *in vitro* or *in vivo* on these parasites, however, *in vitro* and *in vivo* trials of this drug have been studied by many workers on other protozoan and helminth parasites of fishes (**Tojo and Santamarina, 1998a; b; c; Sangmaneedet and Smith, 1999; Shaikh *et al.*, 2003; Shinn *et al.*, 2003**). **Boonyaratpalin and Rogers (1984)** reported 90% reduction of ***P. ambloplitis*** when bass fish (North America) was treated with mebendazole (100 mg / kg body weight / day). Similarly many workers studied *in vivo* effect of mebendazole on secondary ***T. hydatigina***, ***T. ovis*** and ***E. granulosus*** cysts in mice, where severe damage was noticed after prolonged treatment of infected mice with the drug (**Heath *et al.*, 1975, Verheyen, 1982**). Similarly, the time related topographical changes have also been reported in mature metacestode of *T. taeniformis* after the treatment of infected mice with mebendazole (**Borgers *et al.*, 1975; Verheyen *et al.*, 1978**). These changes included gradual disappearance of microtriches and progressive degeneration of the tegument resulting in an irregular surface with grooves, holes and crater formation.

It may be possible that mebendazole acts on the surface membrane and

cause changes in electrochemical gradients which ultimately affect the transport mechanism and is therefore responsible for the shrinkage of the body. It has been reported that benzimidazoles cause the disintegration of microtubules in the syncytial tegument, eventually resulting in tegumental degeneration (**Campbell, 1986**). Mebendazole has been shown to bind with tubulin, which is regarded as a primary mode of action of certain benzimidazole compounds (See references in **Coles, 1983**). Further, mebendazole appears to make larval cestode more vulnerable to attack by the immune reactions of the host as increased number of host cells were found to be attached to the tegument after chemotherapy (**Borgers *et al.*, 1975, Verheyen *et al.*, 1978**).

Rofoxanide produces different types of topographical damage at different concentration on ***A. oreini*** and ***P. kashmirensis***, which could be due to differential uptake as well as distribution of the compound on the parasite surface. This drug causes deeper grooves, lesions, peeling of tegument and coagulation of surface syncitium and thereby may interfere contact digestion through membrane bound enzymes (See **Pappas, 1983**). Deformation of the body and deep lesions has also been reported in ***Gigantocotyle explanatum*** by another salicylanilide drug oxyclozanide (**Ahmad *et al.*, 1987**). It also causes denaturation of the dense coat of mucopolysaccharide which protects the parasite from host secretions (**Marchiondo and Anderson, 1983**).

The halogenated phenols, nitroxynil produce differential effect on

different body regions which could be explained by the existence of a metabolic gradient along the worms as reported in *H. diminuta* **(Coles and Simpkin, 1977)**. This drug caused more damage on scolex and proboscis at 10 and 20 µmole concentrations which have great physiological significance. Thus, it can be speculated that this drug may have similar effect on *A. oreini* and *P. kashmirensis* as mebendazole does in *F. hepatica* **(Rahman *et al.*, 1977)**. Nitroxynil has been reported as uncoupler of oxidative phosphorylation and therefore interfere with the energy metabolism **(Corbett and Gosse, 1971, Hamajima, 1973). Stammers (1975)** has reported by *in vivo* trials that nitroxynil have deleterious affect on the survival, growth, development and morphology of *F. hepatica*. This drug causes histological abnormalities on the testes of the worm which was associated with the uncoupling of oxidative phosphorylation. The inhibitory effect of uncouplers on synthetic processes in anaerobic condition was also correlated by the interference of uncouplers with membrane functions **(Van Den Bossche, 1976)**.

The sulphonamide drug clorsulon was found to cause shrinkage, damage of hooks, deeper groves, peeling of tegument and cracks on the body surface / trunk. This drug is known to inhibit phosphoglycerate kinases and phosphoglucomutase in *Fasciola* and is highly active against adult and immature flukes **(Campbell, 1986)**. At this stage it is difficult to assign any definite mode of action, however, it may be speculated that this drug induces stiffness in the body surface either by interfering with actinomysin complex or

induces depolarization by ionic loss. This certainly requires further biochemical studies to ascertain the exact mode of action.

The carboxylic ionophore monensin induces general shrinkage, lesions, peeling of tegument and blebbings on the scolex, proboscis, body surface and trunk. As a result the scolex / proboscis become deformed. **Rogan and Richard (1986)** have reported blebbings on scolex and soma region of protoscoleces after 24 h *in vitro* treatment with monensin. Swellings of the golgi cisternae in various mammalian tissues and cell lines have also been reported after 10 min to 4 h treatment with monensin **(Griffiths *et al.*, 1983; Tartakoff, 1983; Ellinger and Povelka, 1984)**. Shrinkage of scolex / proboscis may be due to osmotic and ionic disturbances, as this compound has been reported as a metabolite of ***Streptomyces cinnamonensis*** and has capacity to bind Na^+ , K^+ and protons which cause 1:1 cation exchange when inserted into biological membrane **(Tartakoff, 1983)**.

It is evident from the above discussion that different drugs produce different effects on the tegumental surface of ***A. oreini*** and ***P. kashmirensis***. However, the nature and degree of damage induced by the drugs differ possibly because of differences in their biochemical mode of action. But it is emphasized that tegumental damage can be used as a valid parameter for interpreting the *in vitro* action of various anthelmintics. The surface disruption may lead to a variety of secondary metabolic and physiological effects in the parasites. It is therefore, *in vivo* studies are essential to confirm the *in vitro* effects.

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